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Unravelling the Role of Adenosine A_{2A} Receptors on Alpha-Synuclein-Mediated Neurotoxicity

Diana Gabriela Ribeiro Ferreira

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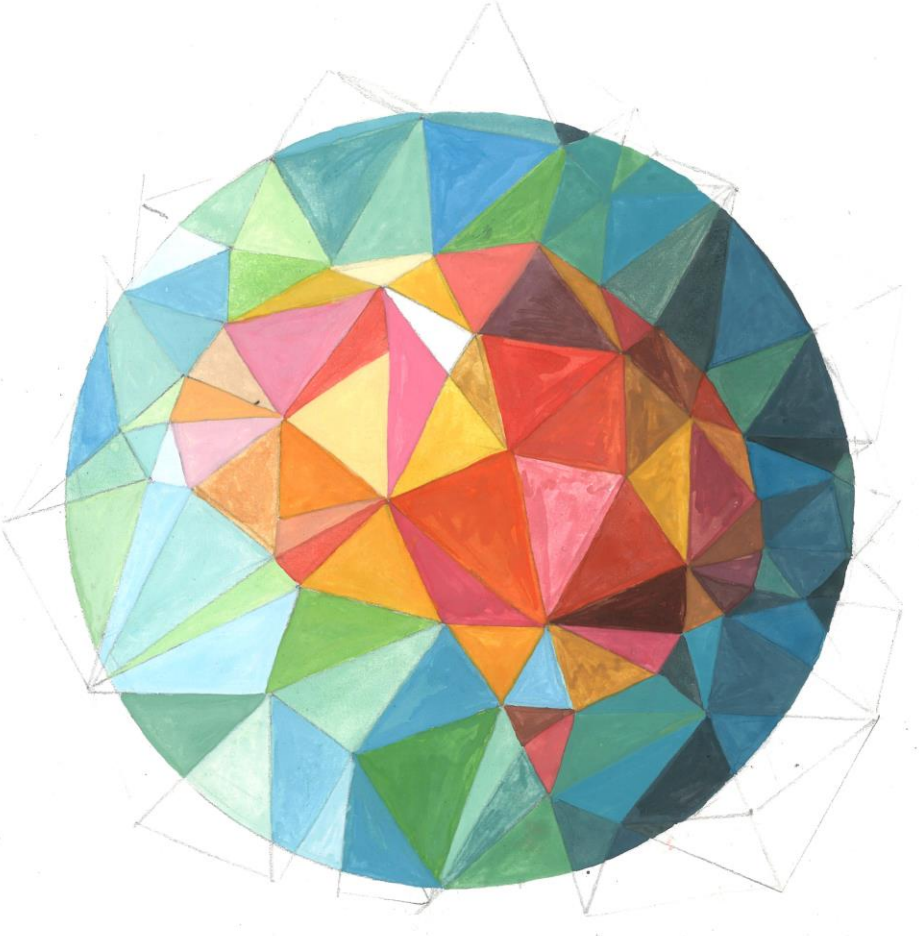

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Dissertação de candidatura ao grau de Doutor em Neurociências apresentada à Faculdade de Medicina da Universidade do Porto

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À minha Mãe e ao meu Pai,
pelo infinito Amor
e por me ensinaram que tudo é possível aos olhos de quem acredita.

Aos meus avós, Bóbó e Bábá,
pelos dias ao som daquele piano e ao sabor do melhor melão.

À nossa querida Tia Cila,
pela eterna memória da verdadeira Páscoa em Família.

Horizonte

O sonho é ver as formas invisíveis
Da distância imprecisa, e, com sensíveis
Movimentos da esperança e da vontade,
Buscar na linha fria do horizonte
A árvore, a praia, a flor, a ave, a fonte -
Os beijos merecidos da Verdade.

Fernando Pessoa, *Mensagem*

The brain is wider than the sky

The brain is wider than the sky,
For, put them side by side,
The one the other will include
With ease, and you beside.

The brain is deeper than the sea,
For, hold them, blue to blue,
The one the other will absorb,
As sponges, buckets do.

The brain is just the weight of God,
For, lift them, pound for pound,
And they will differ, if they do,
As syllable from sound.

Emily Dickinson, *Complete Poems*

Publications

Publications including the scientific content of this dissertation

I. Ferreira DG, Batalha VL, Vicente Miranda H, Coelho JE, Gomes R, Gonçalves FQ, Real JI, Rino J, Albino-Teixeira A, Cunha RA, Outeiro TF, and Lopes LV 2017 Adenosine A_{2A} Receptors Modulate alpha-synuclein Aggregation and Toxicity. *Cereb Cortex* 27(1):718-730. doi: 10.1093/cercor/bhv268.

(Chapter II)

II. Ferreira DG, Temido-Ferreira M, Vicente Miranda H, Batalha VL, Coelho JE, Szegö EM, Marques-Morgado I, Vaz SH, Rhee JS, Schmitz M, Zerr I, Lopes LV, and Outeiro TF 2017 Alpha-synuclein interacts with PrP^C to induce cognitive impairment through mGluR5 and NMDAR2B. *Nature Neuroscience* 20(11):1569-1579. doi: 10.1038/nn.4648.

(Chapter III)

III. Ferreira DG, Outeiro TF, Lopes LV 2017 Review. New Tricks for an Old Target: Synaptic Basis for Cognitive Deficits in Parkinson's Disease. *Frontiers in Neuroscience* (in preparation).

Other publications closely related to the content of the thesis

I. Temido-Ferreira M, **Ferreira DG**, Batalha VL, Marques-Morgado I, Coelho JE, Pereira P, Gomes R, Carvalho S, Canas PM, Cuvelier L, Buée-Scherrer V, Humez S, Schmidt T, Müller CE, Pimentel J, Schiffmann SN, Buée L, Outeiro TF, Bader M, Blum D, Cunha RA, Marie H, Pousinha PA and Lopes LV 2017 Age-related $A_{2A}R$ overexpression in neurons is sufficient to trigger synaptic dysfunction and cognitive deficits (in review).

II. Rivero-Segura NA, Flores-Soto E, Cadena SG Farfán, Coronado-Mares I, Gomez-Verjan JC, **Ferreira DG**, Cabrera-Reyes EA, Lopes LV, Massieu L, and Cerbón M 2017 Prolactin-induced neuroprotection against glutamate excitotoxicity is mediated by the reduction of $[Ca^{2+}]i$ overload and NF-κB activation. *PLoS One* 12(5):e0176910. doi: 10.1371/journal.pone.0176910.

III. Mouro FM, Batalha VL, **Ferreira DG**, Coelho JE, Baqi Y, Müller CE, Lopes LV, Ribeiro JA, Sebastião AM 2017 Chronic and acute adenosine A_{2A} receptor blockade prevents long-term episodic memory disruption caused by acute cannabinoid CB1 receptor activation. *Neuropharmacology* 117:316-327 doi: 10.1016/j.neuropharm.2017.02.021.

IV. Vicente Miranda H, Szegő EM, Oliveira LMA, Darendelioglu E, Breda C, Oliveira RM, **Ferreira DG**, Gomes MA, Rott R, Oliveira M, Munari F, Enguita FJ, Simões T, Rodrigues EF, Heinrich M, Martins IC, Zamolo I, Riess O, Cordeiro C, Ponces-Freire A, Lashuel HA, Santos NS, Lopes LV, Xiang W, Jovin TM, Penque D, Engelender S, Zweckstetter M, Klucken J, Giorgini F, Quintas A, and Outeiro TF 2017 Glycation potentiates alpha-synuclein-associated neurodegeneration in

synucleinopathies. *Scientific Reports* 140(5):1399-1419. doi: 10.1093/brain/awx056.

V. Batalha VL, **Ferreira DG**, Coelho JE, Valadas JS, Gomes R, Temido-Ferreira M, Shmidt T, Baqi Y, Buée L, Müller CE, Hamdane M, Outeiro TF, Bader M, Meijsing SH, Sadri-Vakili G, Blum D, and Lopes LV 2016 The caffeine-binding adenosine A_{2A} receptor induces age-like HPA-axis dysfunction by targeting glucocorticoid receptor function. *Scientific Reports* 6:31493, doi: 10.1038/srep31493.

VI. Coelho JE, Alves P, Canas PM, Valadas JS, Shmidt T, Batalha VL, **Ferreira DG**, Ribeiro JA, Bader M, Cunha RA, do Couto FS, Lopes LV 2014 Overexpression of Adenosine A_{2A} Receptors in Rats: Effects on Depression, Locomotion, and Anxiety. *Front Psychiatry* 67(5):1-8 doi: 10.3389/fpsyt.2014.00067.

VII. Valadas JS, Batalha VL, **Ferreira DG**, Gomes R, Coelho JE, Sebastião AM, Diógenes MJ and Lopes LV 2012 Adenosine A_{2A} receptors mediated neuroprotection is modulated by corticotrophin-releasing factor (CRF) in a model of glutamate induced cell death. *Journal Neurochem*. 123(6):1030-1040 doi: 10.1111/jnc.12050.

Abstract

Parkinson's disease (PD) is a neurodegenerative disorder characterized by a progressive decline in both motor and cognitive functions. Nevertheless, in stark contrast to the nigrostriatal pathology underlying the motor aspects of the disorder, the brain areas involved and the pathophysiological mechanisms underlying cognitive deficits are not fully understood. An important hallmark of PD is the abnormal accumulation of misfolded alpha-synuclein (aSyn)-positive cytoplasmic inclusions termed Lewy bodies (LBs), which occurs early in the disease process and spreads to various brain regions including those related to memory, such as the hippocampus and cortex.

PD's current therapeutic options consist primarily of dopamine replacement strategies that only provide a symptomatic relief of motor symptoms, without affecting cognitive impairment or disease progression. Adenosine A_{2A} receptors ($A_{2A}R$) emerged as promising non-dopaminergic targets for the treatment of PD. Anti-parkinsonian actions are achieved through the blockade of this receptor, whose expression and function become aberrant throughout aging and in age-related pathologies, including the early stages of PD. Although $A_{2A}R$ were originally identified based on their ability to improve PD motor features, they have also been recognized for their beneficial effects on cognition. Moreover, several studies have brought into discussion the potential neuroprotective effect of $A_{2A}R$ antagonists, with epidemiological studies suggesting an inverse correlation between caffeine consumption, an $A_{2A}R$ antagonist, and the risk of developing PD. Furthermore, $A_{2A}R$ deregulation was proposed to play an important role in aSyn-mediated neurotoxicity, since aSyn-induced damage to striatal neurons was clearly reduced in $A_{2A}R$ KO mice.

However, the extent to which $A_{2A}R$ are involved in PD and aSyn-associated toxicity, the underlying protective molecular mechanisms, or the impact on cognitive-associated brain areas are largely unknown; however current data suggest the involvement of mechanisms beyond $A_{2A}R$-D_2R interactions and of brain areas other than the basal ganglia.

This thesis compiles the major findings from my experimental work on the pathological mechanisms underlying PD-associated neurotoxicity and cognitive deficits, and on the ability of $A_{2A}R$ to modulate aSyn-mediated synaptic dysfunction, aggregation, and neuronal death, all of which are key hallmarks in PD and other synucleinopathies.

We report for the first time that, pharmacological $A_{2A}R$ blockade or genetic deletion ($A_{2A}R$ KO mice) fully prevents the aSyn-mediated toxic effects on synaptic plasticity, recorded as long-term potentiation (LTP) in the Schaffer collaterals. This neuroprotective effect afforded by $A_{2A}R$ inhibition is due to the reestablishment of glutamate NMDA receptors (NMDAR) signaling. Furthermore, we also show the ability of $A_{2A}R$ to modulate the latter stages of aSyn-toxicity, using neuroblastoma SH-SY5Y cells overexpressing aSyn and rat primary neuronal cultures exposed to aSyn oligomers for longer periods. Selective $A_{2A}R$ antagonists are able to prevent both exogenous and endogenous aSyn-associated cell death. Interestingly, $A_{2A}R$ blockade also decreases the number of cells displaying aSyn aggregates, raising the possibility that the well-documented effects of $A_{2A}R$ antagonists can involve the control of aSyn aggregation process, thereby preventing the associated neurotoxicity.

We then proposed to explore the mechanisms and potential players involved in aSyn-associated synaptic toxicity and further confirm the instrumental role of $A_{2A}R$ on aSyn pathological processes *in vivo*. We now identify the cellular prion protein (PrP^C) as a key mediator of aSyn-associated synaptic dysfunction. We show that the toxic effects of aSyn oligomers on synaptic function are fully prevented by PrP^C blockade or

deletion, via an NMDAR-dependent mechanism, which we previously reported to be involved in $A_{2A}R$ neuroprotection. Extracellular aSyn oligomers form a complex with PrP^C at the postsynaptic density, inducing the phosphorylation of intracellular Fyn kinase *via* the metabotropic glutamate receptor 5 (mGluR5). aSyn engagement of PrP^C/mGluR5/Fyn signaling causes NMDAR activation and, consequently, dysregulation of Ca^{2+} homeostasis.

Lastly, we show that the *in vivo* blockade of $A_{2A}R$, known to impede mGluR5-evoked phosphorylation of NMDAR, rescues learning and memory deficits, evaluated in the Morris Water Maze and the Y Maze task, and reestablishes synaptic plasticity impairments acompaning early cognitive deficits in a mouse model of PD.

Overall, our findings provide novel and compelling insights into the early mechanisms preceding PD neurodegeneration and identify $A_{2A}R$ and PrP^C as key mediators of aSyn pathology. Furthermore, our results suggest that $A_{2A}R$ represent an important therapeutic target for the development of effective drugs for the treatment of cognitive deficits in PD and other disorders associated with the accumulation of aSyn, thus having immediate implications for clinical practice.

Resumo

A doença de Parkinson (PD) é uma doença neurodegenerativa caracterizada pelo declínio progressivo das funções motora e cognitiva. Contudo, ao contrário dos sintomas motores, que se sabe estarem associados a uma patologia nigroestriatal, as áreas cerebrais envolvidas e os mecanismos fisiopatológicos subjacentes aos défices cognitivos não são conhecidos. Outra importante manifestação anatomopatológica da PD é a acumulação de inclusões citoplasmáticas de alfa-sinucleína (aSyn), denominadas de corpos de Lewy (LBs). Os LBs estão presentes mesmo nas fases iniciais da PD e, propagam-se por várias regiões cerebrais ao longo da progressão da doença, nomeadamente regiões associadas à memória e aprendizagem, como o hipocampo e o córtex.

As opções terapêuticas atuais para PD consistem principalmente em estratégias de reposição de dopamina, que apenas proporcionam uma melhora dos sintomas motores, não alterando os défices cognitivos nem a progressão da doença. Os recetores de adenosina A_{2A} ($A_{2A}R$) surgiram como importantes alvos terapêuticos não dopaminérgicos para o tratamento da PD. As ações anti-parkinsónicas são alcançadas através do bloqueio destes recetores, cuja expressão e função se torna aberrante com o envelhecimento e em patologias associadas, como a PD. Embora o interesse inicial dos $A_{2A}R$ como alvo terapêutico tenha surgido com base na sua capacidade de melhorar os sintomas motores da PD, estes recetores são também reconhecidos pelos seus efeitos benéficos na função cognitiva. Para além disso, vários estudos demonstraram um potencial efeito neuroprotetor dos antagonistas dos $A_{2A}R$, com estudos epidemiológicos sugerindo uma correlação inversa entre o consumo de cafeína, um antagonista dos $A_{2A}R$, e o risco de se desenvolver a PD. Adicionalmente, outro estudo demonstrou que a neurotoxicidade induzida

pela aSyn em neurónios estriatais é prevenida em ratinhos com uma deleção no gene que codifica para os $A_{2A}R$. Contudo, o papel dos $A_{2A}R$ na PD, mais precisamente na toxicidade induzida pela aSyn, os mecanismos moleculares protetores subjacentes ao seu bloqueio e o seu impacto em áreas cerebrais associadas à função cognitiva, não são ainda conhecidos. Esta tese compila as principais descobertas da nossa investigação sobre os mecanismos patológicos subjacentes à neurotoxicidade e aos défices cognitivos associados à PD, e à capacidade dos $A_{2A}R$ em modular estes eventos.

No nosso primeiro estudo demonstramos pela primeira vez que, tanto bloqueio farmacológico dos $A_{2A}R$ como a deleção do gene que codifica para os $A_{2A}R$ (ratinhos $A_{2A}R$ KO), previne a toxicidade sinática induzida por oligómeros de aSyn, medida através da potenciação de longa duração (LTP). Este efeito neuroprotetor é devido ao restabelecimento da sinalização mediada pelos recetores de glutamato NMDA (NMDAR). Adicionalmente, mostramos a capacidade dos $A_{2A}R$ em modular os últimos estágios da toxicidade mediada pela aSyn, usando um modelo celular que sobreexpressa aSyn, e culturas primárias de neurónios expostas a oligomeros de aSyn. Os antagonistas seletivos dos $A_{2A}R$ preveniram a morte celular e neuronal induzida tanto pela aSyn endógena como pelos oligomeros de aSyn adicionados exogenamente. Curiosamente, o bloqueio dos $A_{2A}R$ também teve implicações na agregação de aSyn, diminuindo a percentagem de células com agregados. Estes resultados sugerem que o efeito protetor dos antagonistas dos $A_{2A}R$, descrito já em vários estudos, possa envolver o controle do processo de agregação da aSyn, prevenindo assim a neurotoxicidade associada a esta proteína.

De seguida, fomos investigar os mecanismos e potenciais mediadores envolvidos na toxicidade sináptica associada à aSyn. Identificamos a proteína priónica (PrP^C), na forma celular, como um

mediador chave na disfunção sináptica induzida pela aSyn. Demonstramos que os efeitos tóxicos dos oligómeros na função sináptica são totalmente prevenidos aquando do bloqueio ou deleção da PrP^C. Este efeito ocorre através de um mecanismo dependente dos NMDAR, à semelhança da neuroprotecção associada ao bloqueio dos $A_{2A}R$. Adicionalmente, demonstramos que os oligómeros de aSyn formam um complexo com a PrP^C ao nível da densidade pós-sináptica, levando a uma ativação do recetor de glutamato metabotrópico 5 (mGluR5) que induz a fosforilação da quinase Fyn. A ativação da via de sinalização PrP^C/mGluR5/Fyn leva à fosforilação da subunidade NR2B dos NMDAR e, consequentemente, a uma desregulação na homeostasia de Ca^{2+}.

Concluímos os nossos estudos mostrando que o bloqueio *in vivo* dos $A_{2A}R$, conhecido por prevenir a fosforilação dos NMDAR evocada pelos mGluR5, reestabelece os déficits cognitivos, avaliados através dos testes comportamentais Morris Water Maze e Y-Maze, e danos na plasticidade sináptica observados num modelo de ratinho da PD.

Em suma, as nossas descobertas fornecem dados importantes sobre os mecanismos que precedem a neurodegeneração na PD e identificam os $A_{2A}R$ e a PrP^C como mediadores cruciais na toxicidade associada à aSyn. Para além disso, os nossos resultados sugerem que os $A_{2A}R$ representam um importante alvo terapêutico para o desenvolvimento de fármacos eficazes para o tratamento dos déficits cognitivos associados à PD e outras sinucleinopatías.

Abbreviation List

1-Naphtlyl PP1	1-(1,1-Dimethylethyl)-3-(1-naphthalenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine, Src-family inhibitor
5'NT	5'-nucleotidase
5-HT	5-Hydroxytryptamine
6-OHDA	6-Hydroxydopamine
A_{2A}R	Adenosine A_{2A} receptors
AAV2-hAADC	Adeno-associated virus encoding human aromatic L-amino acid decarboxylase
AC	Adenylate cyclase
aCSF	Artificial cerebrospinal fluid
AD	Alzheimer's disease
ADA	Adenosine adeaminase
ADK	Adenosine kinase
ALS	Amyotrophic lateral sclerosis
AMPK	AMP-activated protein kinase
ANS	Autonomic nervous system
APV	DL-2-Amino-5-phosphonopentanoic acid, NMDAR-selective antagonist
AR	Adenosine receptors
aSyn	Alpha-synuclein
ATP13A2	ATPase type 13A2
Aβ	Amyloid-beta
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
Caff	Caffeine
CaM	Calmodulin
CAMKII	Calcium/Calmodulin-Dependent Protein Kinase II
cAMP	Cyclic 5'-adenosine monophosphate
CGS 21680	4-[2-[[6-Amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzenepropanoic acid

CHCHD2	Coiled-coil-helix-coiled-coil-helix domain containing 2
CNS	Central nervous system
CNT	Concentrative nucleoside transporters
COMT	Cathecol-o-methyl-transferase
CoQ10	Coenzyme Q10
CREB	cAMP response element binding protein
DA	Dopamine
DARPP-32	Dopamine- and cAMP-regulated protein with molecular weight of 32 kDa
DBS	Deep brain stimulation
DCTN1	p150Glued subunit of dynactin
DGNF	Glial cell line-derived neurotrophic factor
DHPG	(S)-3,5-Dihydroxyphenylglycine, mGluR5-selective agonist
DIV	Days *in vitro*
DJ-1	Daisuke-Junko-1
DLB	Dementia with Lewy bodies
DMSO	Dimethylsulphoxide
Dox	Doxycycline
DTT	Dithiothreitol
EIF4G1	Eukaryotic translation initiation factor 4-γ1
ENS	Enteric nervous system
ENT	Equilibrative nucleoside transporters
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FBX07	F-box only protein 7
fEPSP	Field excitatory postsynaptic potential
GABA	Gamma-aminobutyric acid
GBA	Glucocerebrosidase
GPCR	G-protein coupled receptors
GPi	*Globus palidus internus*
GSH	Glutathione
GWAS	Genome-wide association studies
HCY	Homocysteine

HD	Huntington’s disease
HFS	High-frequency stimulation
Ka	Dissociation constant or affinity constant
KO	Knockout
KW-6002	(E)-8-(2-(3,4-dimethoxyphenyl)-vinyl)-1,3-diethyl-7-methyl-3,7-dihydropurine-2,6-dione, istradefylline, A_{2A}R-selective antagonist
LB	Lewy body
L-DOPA	L-dihydroxyphenylalanine
LN	Lewy neurite
LRRK2	Leucine-rich repeat kinase 2
LTP	Long-term potentiation
MAO-B	Monoamine oxidase B
MCI	Mild cognitive impairment
MDS	Movement Disorder Society
MPEP	2-Methyl-6-(phenylethynyl)pyridine, mGluR5-selective antagonist
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Motor symptoms
MSA	Multiple system atrophy
Neo	Neomycin
NMDA	N-Methyl-*D*-aspartate
NMDAR	NMDA receptors
NMS	Non-motor symptoms
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PD	Parkinson´s disease
PI	Propidium iodide
PINK1	PTEN-induced putative kinase 1
PKA	Protein kinase A
PKC	Protein kinase C
PNS	Peripheral nervous system
PVDF	Polyvinylidene difluoride
qPCR	Quantitative real-time PCR

rAAV	Recombinant adeno-associated virus
RBD	REM behaviour disorder
REM	Rapid eye movement
RIPA	Radio-immunoprecipitation assay
ROS	Reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute medium
SAH	S-adenosyl-homocysteine;
SAHH	S-adenosyl-homocysteine hydrolase
SCH 58261	5-Amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SN	*Substantia nigra*
SNc	*Substantia nigra pars compacta*
STN	Subthalamic nucleus
STR	Staurosporine
SYN-115	Tozadenant
TBS-T	Tris-buffered saline containing 0.1% tween 20
Tg	Transgenic
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VPS35	Vacuolar protein sorting 35
WO	Weeks old
Wt	Wild-type
XO	Xanthine oxidase
ZM 241385	4-(2-[7-Amino-2-(2-furyl)[1,2,4]triaolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol

Table of Content

Chapter I

Introduction

State of the art

Over the past decades, the average life expectancy has increased globally, reaching a worldwide average of about 71.4 years in 2015 and around 80 years in developed countries (World Health Organization, 2016). This advance has been achieved especially due to improvements in sanitation, medical improvements, rising living standards, and a decline in child mortality. Considering the demographics of the world population, between 2000 and 2050 the proportion of people over 60 years will double from about 11% to 22%, which, in absolute terms, means an increase from 605 million to 2 billion people (Jin et al., 2014). Although the increasing life expectancy generally reflects positive human development, new challenges are arising, especially in the health sector due to aging-related diseases such as neurodegenerative diseases.

Neurodegenerative diseases, such as Parkinson's disease (PD), are devastating progressive conditions and a major healthcare challenge worldwide, disabling millions. Although there are palliative treatments, currently, there is no cure or effective disease-modifying therapy for any of these disorders.

Hence, major efforts must be directed toward the identification of the molecular players responsible for disease onset and a better understanding of the pathological mechanisms involved, in order to open the gates to novel and more effective therapies.

Parkinson's disease

Parkinson´s Disease as a Systemic Disease

200 years have gone since James Parkinson´s wrote a monograph entitled *An Essay on the Shaking Palsy* (Goetz, 2011; Parkinson, 2002), in which he detailed the symptoms of *paralysis agitans*, a condition he had observed among six of his patients and that 60 years later would be named after him – (Charcot et al., 1887):

> *"Involuntary tremulous motion with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forward, and to pass from a walking to a running pace"*

The essay focused mainly on the motor features of PD and, in fact, PD was originally considered as a movement disease characterized mainly by its motor symptoms (MS), such as bradykinesia or slowness of movement, resting tremor, rigidity, and postural and gait instability (Fahn, 2003; Lees et al., 2009). Based on this, PD was often considered to involve a simple pathological process comprising the selective loss of dopaminergic neurons from the *substantia nigra* pars *compacta* (*SNc*), a small region in the mesencephalon. These dopaminergic neurons innervate the basal ganglia, and their selective degeneration and consequent reduction of striatal dopamine (DA) concentration, directly alter the activity of the cortico-striato-pallido-thalamocortical pathways controlling motor behaviour and action selection (**Fig. 1.1**; Wichmann and DeLong, 2003).

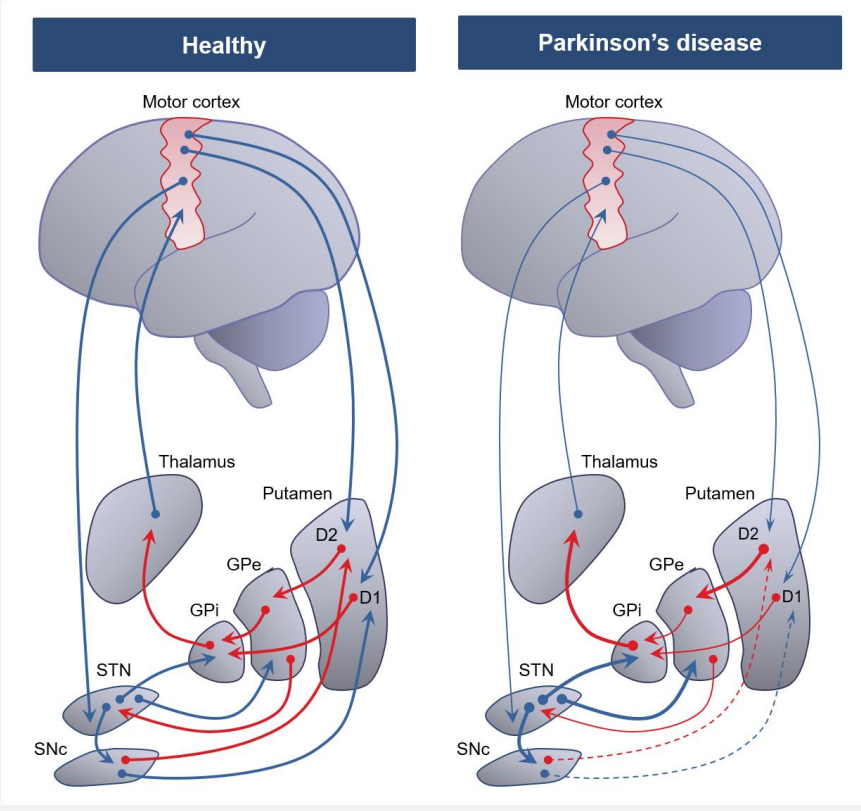


Figure 1.1. Schematic diagram of the direct and indirect pathways of the basal ganglia motor circuits in healthy and PD states. Red arrows indicate inhibitory projections, and blue arrows indicate excitatory projections. The changes in the thickness of the arrows in the PD state indicate the proposed increase (larger arrow) or decrease (thinner arrow) in firing rate activity of specific connections. The dashed arrows used to label the dopaminergic projection from the *SNc* to the putamen in PD indicate a partial lesion of that system in this condition. Note that many connections have been purposefully omitted from this diagram. *GPe*, *globus pallidus*, external segment; *GPi*, *globus pallidus internus*; SNc, *substantia nigra pars compacta*; STN, subthalamic nucleus.

Recently, there has been a shift in the conceptualization of the disease, and PD is now recognized as a systemic disorder affecting several distinct neuronal populations of the central and peripheral nervous system (CNS; PNS), which leads to a high range of clinical features. Even though MS have historically been identified as the most relevant aspect of the disease (Parkinson, 2002), accumulating clinical evidence reveals a broad spectrum of non-motor symptoms (NMS) associated with PD. Furthermore, it is increasingly clear that, at least some of these non-parkinsonian features, can predate the onset of the classical motor signs, by years and even decades (Berg et al., 2015; Chaudhuri et al., 2006).

NMS occurring in PD cover a wide variety of manifestations, including autonomic dysfunction (gastrointestinal and cardiovascular dysfunction with orthostatic hypotension), sensory disturbances (olfactory deficits, also known as hyposmia, and vision problems), pain, sleep disorders, neuropsychiatric problems (depression and anxiety) and cognitive impairment (**Figure 1.2**; Chaudhuri and Schapira, 2009; Hawkes et al., 2010; Lim et al., 2009; Savica et al., 2010; Schapira and Tolosa, 2010; Tolosa et al., 2009). Cognitive impairment is an important feature of PD with a spectrum of deficits ranging from none to severe dementia. The intermediate zone between normal cognition and dementia has been termed mild cognitive impairment (MCI; Dalrymple-Alford et al., 2011; Litvan et al., 2012). These cognitive symptoms involve abnormalities in spatial performance and memory deficits, with both short- and long-term memory being affected. Alterations in organization, planning, regulation of goal-directed behaviours, information retrieval and attention are widely observed in PD patients and are key events triggering the manifestations of PD-associated cognitive decline (Aarsland et al., 2003; Chahine et al., 2016; Jellinger, 2017). Epidemiological studies show that 18.9% to 38.2% of PD patients develop MCI in the early stages of the disorder (Litvan et al., 2011, 2012; Williams-Gray et al., 2007), and up to 50% of those with

PD develop dementia after 10 years, increasing to over 80% after 20 years (Bosboom et al., 2004; Hely et al., 2008; Williams-Gray et al., 2007, 2013).

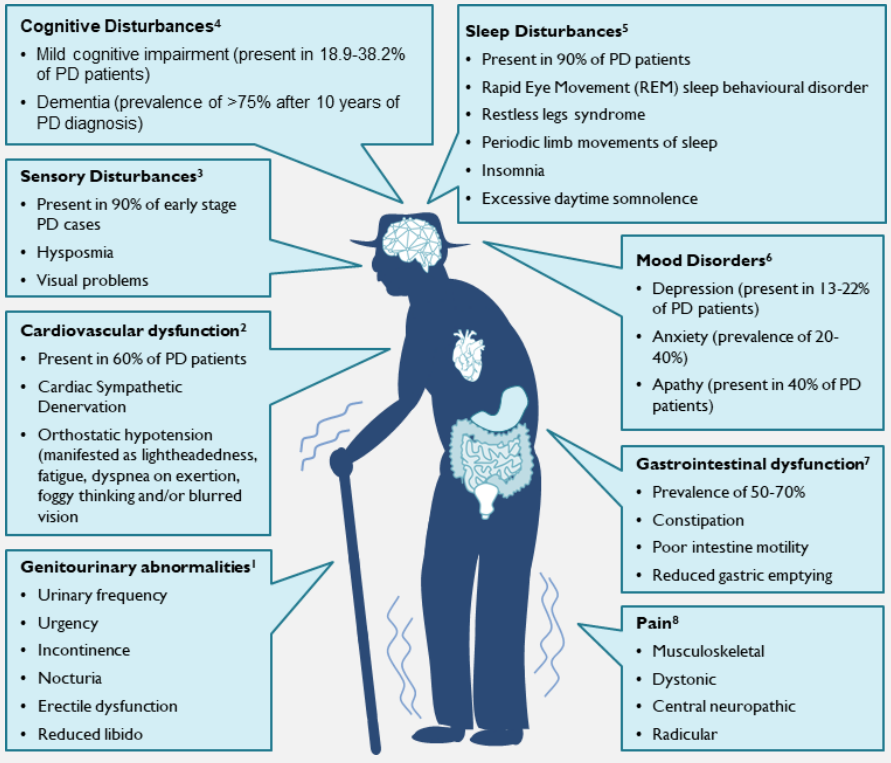


Figure 1.2. Schematic diagram of NMS in PD. PD NMS reflect deficits in various functions of the CNS and autonomic nervous system (ANS). Multisystem involvement develops to varying levels of severity and in a variable sequence in different patients. [1]Bronner et al., 2015; Sakakibara et al., 2016; Winge, 2015; [2]Pfeiffer, 2016; [3]Zhu et al., 2016; [4]Zweig et al., 2016; [5]Albers et al., 2017; [6]Goldman, 2016; [7]Sung and Nicholas, 2013; [8]Valkovic et al., 2015.

Characterization of NMS is potentially valuable for PD early identification since the current diagnosis of PD requires multiple MS to be established, and while subtle motor signs may be present, a clinical diagnosis cannot be made until they become more definite. Nonetheless, at the time the diagnosis is made, PD has progressed over an estimated period of 10 to 15 years and a loss of 50 to 60% of the nigral DA neurons already occurred (**Fig. 1.3**). This limits all the current potential therapeutic interventions, especially those aiming at neuroprotection.

Based on this problematic, the Movement Disorders Society (MDS) has recently established a task force to redefine clinical PD considering the new insights of the disease (Berg et al., 2015, 2015; Postuma et al., 2015). MDS proposed that early PD should be divided into three stages (**Fig. 1.3**):

(I) Preclinical PD – the neurodegenerative processes have already started, but there are no evident symptoms or signs (defined by disease biomarkers when available);

(II) Prodromal PD – presence of early symptoms and signs, but are still insufficient to define the disease;

(III) Clinical PD – the diagnostic criteria for PD are fulfilled based on the presence of classical MS.

Nevertheless, there is no validated early diagnostic biomarkers of PD. Although non-specific, precursor NMS such as constipation, depression, mild cognitive impairments, hyposmia, or rapid eye movement (REM) sleep disorder (RBD) characterize a population in which future biomarkers for the diagnosis of prodromal PD should be tested (Miller and O'Callaghan, 2015). This would be of great value and allow (1) to intervene at the onset or in an early phase of the disease and (2) to monitor the progress of therapeutic interventions that may slow or stop PD course.

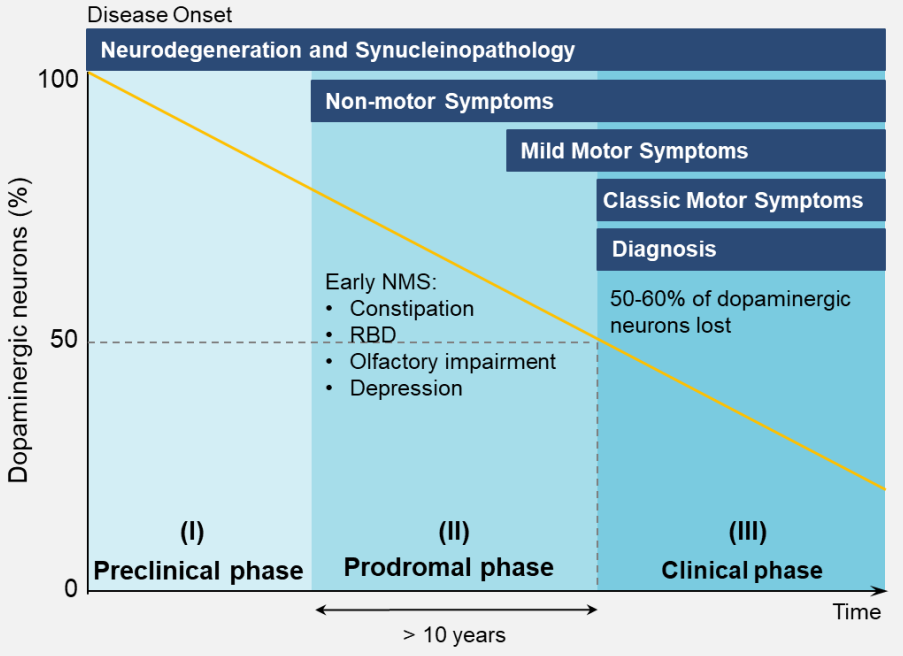


Figure 1.3. Graphical illustration of PD progression. The yellow line represents the continuous degeneration of the dopaminergic neurons of the *SN*. People at risk of PD progress to a preclinical phase before the prodromal phase, in which nonmotor symptoms and slight motor signs can occur, and finally the clinical phase. The prodromal phase with early neurodegenerative changes occurs years or even decades before the clinical diagnosis of PD can be made.

Overall, the shift in focus from the classic MS to NMS has, therefore, followed the strong evidence that: (1) several NMS begin with an earlier pre-motor prodrome; (2) at the time MS appear, approximately 50 to 60% of dopaminergic neurons have already been lost, and; (3) under chronic dopaminergic treatment the progression of MS stabilizes in most people with long-term disease; however, NMS, such as cognitive impairments, are not affected by dopamine replacement therapies and continue to develop throughout the course of the disease, accumulating

greater disability. In fact, several studies show that cognitive impairments and clinically evident dementia severely affect patient's global health, and their behavioral manifestations have disruptive effects on familial and social dynamics, which may result in nursing home placement and, finally, cause a severe negative impact on patient quality of life and caregivers burden (Aarsland et al., 2007, 2008; Levy et al., 2002; Marras et al., 2008). This evidence reveals a strong need for studies on the pathological mechanisms of the NMS in order to understand the pathological process of the disorder and, consequently, find the urgently required biomarkers and therapeutic strategies to tackle PD-associated cognitive dysfunction and dementia.

Epidemiology of Parkinson's Disease

Epidemiological data regarding the prevalence, number of cases, incidence, and number of newly diagnosed cases of PD are of interest for their potential to identify risk factors and improve understanding of the disease's natural history. These data have also been increasingly used to guide effective planning of medical services. Although several studies report data on the epidemiology of PD, methodological differences between studies make a direct comparison of prevalence and incidence estimates difficult.

PD is, at present, the second most common neurodegenerative disorder in the elderly population, after Alzheimer´s disease (AD; Dorsey et al., 2007), with a generally accepted prevalence ranging from 35.8 to 12.500 *per* 100.000 inhabitants. Age is the greatest risk factor for the development of PD, with a nearly exponential increase in incidence, and consequently prevalence, after 55 years of age (Driver et al., 2009; Pringsheim et al., 2014). This trend has important public health implications; with an aging population and rising life expectancy

worldwide, the number of people with PD is expected to increase by more than 50% by 2030 (Dorsey et al., 2007).

PD seems more frequent in Europe, North America, and Australia, with a prevalence of 1601, compared with individuals from Asia, where the prevalence is 646 (*per* 100.000). PD is also more prevalent in males (1729 *per* 100.000, >65 years) than in females (1644 *per* 100.000; Moisan et al., 2016; Riedel et al., 2016). The annual incidence estimates range from 1,5 to 346 *per* 100.000 inhabitants (von Campenhausen et al., 2005; Muangpaisan et al., 2009; Zou et al., 2015). It is important to note that incidence studies may be underestimated since they are affected by under-diagnosing of PD, especially among the most elderly.

Aetiology of Parkinson's Disease

Despite the many decades of research, our understanding of the aetiology of PD, like many other human neurodegenerative disorders, is still very scarce due to the difficulty to infer an active sequence of events, often expressed over prolonged periods of time. Current theories suggest a combination of genetic predispositions, complemented potentially by epigenetic, and environmental factors to be involved in PD aetiology (**Fig. 1.4**; Delamarre and Meissner, 2017; Wirdefeldt et al., 2011).

Genetics. Over the past 2 decades, there has been an explosion of research on the genetics of PD. Although most cases of PD are thought to be sporadic (> 90%), genetics likely plays a significant role (Lill, 2016). Family members of affected patients have a 2- to 3-fold increased risk to develop the disease, compared to subjects in the general population (Savica et al., 2016; Sveinbjörnsdottir et al., 2000). Rare familial forms of PD with both autosomal dominant and recessive inheritance have been described. Several genes have been associated with monogenic forms of

the disease, with *SNCA* (encoding for alpha-synuclein; Polymeropoulos et al., 1997), *LRRK2* (leucine-rich repeat kinase 2; *PARK8*; Paisán-Ruíz et al., 2004; Zimprich et al., 2004), *VPS35* (vacuolar protein sorting 35; Vilariño-Güell et al., 2011; Zimprich et al., 2011), *CHCHD2* (coiled-coil-helix-coiled-coil-helix domain containing 2; Jansen et al., 2015), *DCTN1* ($p150^{Glued}$ subunit of dynactin; Araki et al., 2014), and *EIF4G1* genes (eukaryotic translation initiation factor 4-γ1; Chartier-Harlin et al., 2011), causing autosomal dominant forms of PD, and *PINK1* (PTEN-induced putative kinase 1; *PARK5*; Bonifati et al., 2005), *DJ-1* (Daisuke-Junko-1; *PARK7*), *parkin* (*PARK2*; Djarmati et al., 2004), *ATP13A2* (ATPase type 13A2; *PARK9*; Ramirez et al., 2006), *PLA2G6* (Paisan-Ruiz et al., 2009), and *FBX07* genes (F-box only protein 7; *PARK15*; Di Fonzo et al., 2009) associated with recessive transmission.

In addition to monogenic forms of PD, which, as mentioned, only account for a small proportion of all PD cases, advances in genomics and bioinformatics have uncovered additional genetic risk factors for PD. In the past decade, 900 genetic association studies, such as genome-wide association studies (GWAS), have identified more than 30 loci associated with the modulation of PD risk (Nalls et al., 2014). Of note, most of the GWAS hits appear to be driven by non-coding variation and are thus likely associated with the regulation of gene expression (Nalls et al., 2014). Heterozygous *glucocerebrosidase* gene (*GBA*) mutations, responsible for the most frequent lysosomal storage disorder, Gaucher disease, were shown to increase the risk of developing PD more than 5-fold (Lees et al., 2009). One of the strongest associations with the disease is alpha-synuclein (aSyn), a small protein preferentially found at presynaptic termini and in the nucleus (Maroteaux and Scheller, 1991). There are pathogenic missense mutations and multiplication mutations in *SNCA* gene encoding for aSyn. Thus, not only a mutant protein, but also both differences in *SNCA* expression and, consequently, wild-type (wt) aSyn protein levels

have been shown to play a critical role in PD susceptibility, as demonstrated in families with *SNCA* triplications and duplications (Kalia and Lang, 2015; Miller et al., 2004; Mutez et al., 2011). The role of aSyn in PD will be further dissected.

Epigenetics. Epigenetic modifications provide phenotypic plasticity, allowing adaptation to a change in the environment without modifying the genotype. Based on this, recent studies showed that several regulatory mechanisms such as DNA methylation of promoter regions, histone modifications, and RNA-based mechanisms are involved in PD-related gene expression (Ammal Kaidery et al., 2013; Labbé et al., 2016). Epigenetic methylation of the *SNCA locus*, which is associated with a decrease in gene expression, was shown to be reduced in
DNA from sporadic PD patients *SN*, putamen and cortex (Jowaed et al., 2010), pointing toward a yet unappreciated epigenetic regulation of *SNCA* expression in PD. In accordance, *post-mortem* analysis of PD patients brains also revealed a significantly decrease in *SNCA* methylation in the *SN* (Matsumoto et al., 2010). Similarly, sporadic PD patients have been shown to have differential expression of various miRNA probes, including miR-34b/c, which has been associated with the development of mitochondrial dysfunction (Margis et al., 2011; Miñones-Moyano et al., 2011).

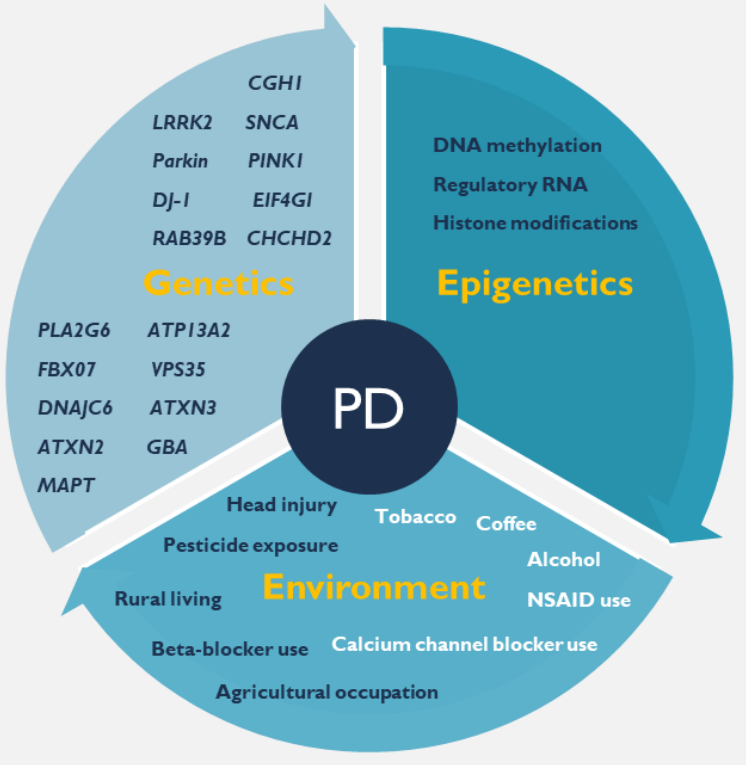


Figure 1.4. Schematic overview of the interplay of genetic, environmental and epigenetic domains possibly underlying Parkinson's disease (PD) pathophysiology. Results of epidemiological studies have revealed various environmental exposures that increase (in white) or decrease (in blue) the risk of developing PD. Findings of genome-wide association studies have identified genetic risk factors, which are polymorphisms within certain genes that influence risk for developing PD. Epigenetic modification, which alters the functionality of a *locus* or chromosome without changing the underlying DNA sequence, may account for some of the fundamental differences in expression patterns between PD patients and controls. Examples of relevant risk factors in each domain are listed.

Environment. Occupational, lifestyle and environmental factors, possibly in interaction with each other or with susceptibility genes, also play an important part in influencing the general risk of developing PD (**Fig. 1.4**). Epidemiologic studies have shown an increased risk of PD with pesticide exposure, prior head injury, rural living, β-blocker use, agricultural occupation, and wood preservative use. Among specific toxins that may contribute to PD are 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and other isoquinoline derivatives, organophosphate, pesticides, and possibly mitochondrial toxins such as rotenone. Several neurotoxins not only produce a PD phenotype being used in many PD animal models of the disease, but can also affect different epigenetic mechanisms, such as methylation, demethylation, hyperacetylation, and deacetylation of certain genomic regions (Labbé et al., 2016). Moreover, an interesting inverse relationship has been reported between PD and coffee drinking, tobacco smoking, non-steroidal anti-inflammatory drug use, calcium channel blocker use, or alcohol consumption (Noyce et al., 2012).

The risk of developing PD is clearly multifactorial, but the elaborate interplay between the several factors is just beginning to be deciphered. A further understanding of PD risk factors and their interactions is expected to have broad implications for the elucidation of the disease pathogenic mechanisms, identification of biomarkers, and new therapeutic interventions.

Lewy Body Pathology and Cognitive Deficits

Almost 100 years after Parkinson had published his findings, the German neurologist Friedrich Lewy identified intracytoplasmic neuronal protein deposits in the brain of PD patients, which later became known as Lewy bodies (LBs; Lewy, F. H., 1912). However, the biochemical nature of the deposits remained unknown until 1997, when Maria Spillantini and

colleagues found that their main component was the presynaptic protein aSyn (Spillantini et al., 1997). These aSyn deposits can also be found in neuronal processes (Lewy neurites - LN) as well as in astrocytes and oligodendroglial cells of PD patients (Kalia and Lang, 2016; Spillantini et al., 1997). In addition, LB brain pathology is also found in other two neurodegenerative disorders, dementia with LBs (DLB) and multiple system atrophy (MSA), which are now collectively termed as synucleinopathies (Baba et al., 1998; Wakabayashi et al., 1998a). Despite the fact that all these disorders have aSyn-containing inclusions deposited in the brain, the cell types and brain structures that are affected can vary between them. This leads to different clinical manifestations, which are the basis for the differential diagnosis. However, these disorders also share several symptoms such as chronic and progressive decline in motor, cognitive, behavioral, and autonomic functions (Halliday et al., 2011). Recently, a new definition and diagnostic criteria for clinical PD was published (Postuma et al., 2015). Under the light of these new criteria, early dementia is no longer considered an exclusion criterion for PD, eliminating the distinction between PD and DLB. As so, DLB can be now classified as a PD subtype if all other criteria for PD are met (Kalia and Lang, 2016).

In contrast to MS, which are thought to reflect neuronal loss within the *SN* (**Fig. 1.1**), little is known about the functional networks underlying NMS in PD, although the depletion of striatal DA has also been associated with it (Green et al., 2002). Besides the dopaminergic transmission abnormalities, several studies indicate that the accumulation of LBs and LNs (Spillantini et al., 1997) is not restricted to the *SN*, but can also be found in extranigral regions, such as cortex, amygdala, *locus coeruleus*, reticular formation, *vagus nuclei*, and the hippocampus, as well as outside of the brain, namely in the spinal cord and PNS (Beach et al., 2010; Del Tredici et al., 2010; Forno, 1987). As mentioned, several

neuropsychological symptoms, such as cognitive impairments and dementia, have a relatively poor response to dopaminergic therapy, suggesting that dysfunction of these extranigral neuronal populations and possibly LB pathology underlie, or at least contribute to, the appearance of these symptoms (Chaudhuri et al., 2006).

According to the Braak theory, LB pathology progresses in a stereotyped pattern over the course of PD, starting in the olfactory mucosa and enteric nervous system (ENS), traveling into the brain via vagal and olfactory nerves, and progressing to particular structures in a caudal-to-rostral predictable direction within the brain. This pattern begins in the dorsal motor nucleus of the glossopharyngeal and vagal nerves and olfactory bulb (stage one), ascending to the pontine tegmentum (stage two), midbrain, basal forebrain and limbic system (stage three), mesocortex and allocortex (stage four), and finally invading the neocortex at the latest stages of the disease (stage five and six; Braak et al., 2003, 2004). The presence of LBs occur early in the disease process and is accompanied by progressive neuronal dysfunction and eventually death of affected neuronal populations (Braak and Del Tredici, 2008). Moreover, the proposed temporal and spatial progression seems to explain the clinical course of PD. Evidence for an association between LB pathology and NMS is most convincing for cognitive impairments in PD. *Post-mortem* studies have shown a strong correlation between cognitive impairments, including dementia, and subcortical and cortical LB formation, including the transentorhinal and entorhinal cortices, hippocampus, other limbic cortex regions, and neocortex (Beach et al., 2009; Braak et al., 2005; Caviness et al., 2011; Irwin et al., 2012; Kempster et al., 2010; Kövari et al., 2003). Accordingly, a recent clinical study also reported a correlation between LB and several NMS, including cognitive impairments, in *LRRK2*-related PD (Kalia et al., 2015).

Structural brain imaging allows *in vivo* determination of regional neurodegeneration. PD studies, using a range of imaging analyses, have reported an association between regional brain volumes, namely parietal-temporal cortex, prefrontal cortex, hippocampus and amygdala, and cognitive decline (Ibarretxe-Bilbao et al., 2008; Lyoo et al., 2010; Song et al., 2011). In fact, many PD patients present an Alzheimer's disease (AD) pattern of brain atrophy together with neuropathological changes in the hippocampus (Apaydin et al., 2002; Compta et al., 2011; Farlow and Cummings, 2008; Kang, 2016; Weintraub et al., 2012). Interestingly, a mice model of PD overexpressing human wt aSyn, the main component of the LB, develop neuronal degeneration not only in the *SN* but also in the hippocampus, as well as reduced hippocampal neurogenesis, leading to progressive motor decline and cognitive impairment (Nuber et al., 2008). Consistently, hippocampal and parietal lobes atrophy has also been suggested to predict a more rapid future cognitive decline (Weintraub et al., 2012). These findings support the involvement of the hippocampus with cognitive impairment and long-term decline in PD.

aSyn, a key player in Parkinson's Disease

A major breakthrough in PD pathogenesis was made when, in 1997, a mutation in *SNCA* was identified in an Italian family and in three unrelated families of Greek origin with autosomal dominant PD (Polymeropoulos et al., 1997). It was of considerable importance that the pathology was essentially identical to that observed in sporadic PD; the presence of LBs and neuronal degeneration was then confirmed in many regions of the brain, including the *SN* and *locus coeruleus*, in the Italian family (Golbe et al., 1990). In the same year, several immunohistochemical studies described fibrillar aggregates of aSyn as the major protein component of LBs in PD and also in DLB and MSA (Arima et al., 1998; Baba et al.,

1998; Galvin et al., 2001; Irizarry et al., 1998; Spillantini and Goedert, 2000; Spillantini et al., 1997; Takeda et al., 1998; Wakabayashi et al., 1997, 1998b; Waxman and Giasson, 2009). This suggested, for the first time, that aSyn was not only involved in the aetiology of PD but also underlaid LB formation in this condition and in other synucleinopathies. Since then, many research efforts have been concentrated on aSyn. To date, six missense mutations (A30P, E46K, A53T, A53E, H50Q, G51D) in the *SNCA* gene have been shown to be associated with autosomal dominant forms of the disease (**Fig. 1.5**; Appel-Cresswell et al., 2013; Krüger et al., 1998; Lesage et al., 2013; Pasanen et al., 2014; Polymeropoulos et al., 1997; Zarranz et al., 2004).

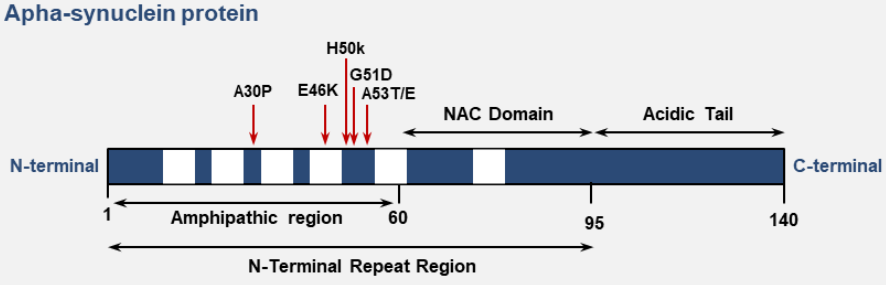


Figure 1.5. Schematic representation of the different domains of aSyn. aSyn is divided into three overlapping regions, namely an N-terminal repeat region (aa 1–95), a central hydrophobic NAC region (aa 61–95), and an acidic C-terminus (aa 96–140). The repeat region contains six conserved repeat motifs (white boxes) and is believed to be involved in aSyn interactions; the NAC domain is required for the aggregation of aSyn, whereas the acidic tail in the C-terminus is critical for binding of various ions and proteins. All six PD-related mutations are located within the amphipathic region in the N-terminus of the protein.

The next landmark studies linking *SNCA* to PD, identified triplications (Singleton et al., 2003) and duplications (Chartier-Harlin et al., 2004; Ibáñez et al., 2004) of the *SNCA locus* in separate families with an autosomal dominant inheritance pattern, which was later associated with an increase in total aSyn levels (Miller et al., 2004). Several studies show that the simple increase in the expression levels of the wt protein is sufficient to cause neurodegeneration and that such increase may underlie the pathogenesis of sporadic PD. Besides, *SNCA* dosage correlates with earlier onset, faster progression, and more severe disease presentation (Konno et al., 2016; Ross et al., 2008). Nevertheless, the exact cause of aSyn abnormal accumulation, neuronal death and disease progression related to its overexpression remains largely unknown (Imai et al., 2001; Janicki and Monteiro, 1999; Ko et al., 2008; Morishima et al., 2001; Saha et al., 2000; Shimura et al., 2001; Wu et al., 2000).

When a specific genetic defect is identified in rare familial cases, it is almost a reflex reaction to look at the same gene in the sporadic disease, with the hope of identifying polymorphisms that may be linked to the disease in association studies. In fact, the contribution of aSyn in sporadic forms of the disease was further strengthened by GWAS showing that polymorphic variations in non-coding regions of the *SNCA locus* represent a major risk factor for sporadic PD pathogenesis (Edwards et al., 2010; Satake et al., 2009; Simón-Sánchez et al., 2009). Specific variations, including a dinucleotide repeat sequence REP1 within the *SNCA* promoter, rs356168 in intron 4, and rs356210 in the 3′ UTR region, have all been proposed to influence both *SNCA* expression and disease risk (Fuchs et al., 2008; Maraganore et al., 2006; Soldner et al., 2016). These studies, support a role for aSyn in the aetiology of both familial and sporadic forms of this disease and extend the relevance of this protein to a larger cohort of patients (Hamza et al., 2010; Satake et al., 2009; Simón-Sánchez et al., 2009).

aSyn is a ubiquitous protein highly expressed in the brain. This protein is abundant in neurons, especially enriched in presynaptic terminals (Goedert, 1999; Lavedan, 1998) and, although its exact function is unknown, it is thought to be involved in synaptic function and plasticity, processes highly influenced by aging. It is prominently expressed in the cortex and hippocampus, brain regions with a high synaptic plasticity potential (Maroteaux and Scheller, 1991). It has also been postulated that aSyn regulates the size of the presynaptic vesicular pool (Murphy et al., 2000), neurotransmitter release and processes associated with the organization and regulation of synaptic vesicles (Kahle et al., 2000; Perez et al., 2002). Delayed developmental expression of aSyn compared to synaptophysin, suggests its role in maintenance rather than formation of synapses (Murphy et al., 2000; Withers et al., 1997).

aSyn is a 140-amino acid soluble protein that is frequently divided into three overlapping regions: the N-terminal repeat region of about 100 amino acids containing six 11-residue repeats some of which display amphipathic properties; the hydrophobic NAC region from amino acids 61–95; and the acidic C-terminal region (George et al., 1995; Lundvig et al., 2005). aSyn displays a high degree of structural plasticity that is governed by its environment. Under normal physiological conditions, aSyn is intrinsically unfolded in solution, which means that in the purified form at neutral pH it lacks an ordered secondary or tertiary structure. Upon binding to phospholipid membranes or synthetic vesicles containing acidic phospholipids, its N-terminal repeat region acquires a α-helical structure that is not prone to aggregation (Bartels et al., 2011; Deleersnijder et al., 2013). However, aSyn exhibits a propensity to misfold into protofibrils and higher-order oligomers following changes in pH and ionic strength, increases in molecular crowding, and interactions with lipid membranes as well as secondary modification such as DA adduction, nitrosylation, and phosphorylation (Conway, 2001; Conway et al., 1998, 2000; Ding et al.,

2002; Fink, 2006; Hashimoto et al., 1999; Kowall et al., 2000; Perrin, 2001; Sharon et al., 2003; Shtilerman et al., 2002; Tsigelny et al., 2008; Vila et al., 2000; Volles and Lansbury, 2002; Volles et al., 2001). It is speculated that distinct strains of pathological aSyn likely exist in neurodegenerative disease brains and may underlie the tremendous heterogeneity of synucleinopathies. aSyn aggregates first into multiple soluble oligomeric species, ranging from dimers to more than a 70-monomers assembly (Cremades et al., 2012), that can be stabilized by β-sheet-like interactions. This β-sheet-like structure can readily polymerize into higher molecular weight insoluble amyloid-like fibrils that are the main components of the LBs and LNs (**Fig. 1.6**; Braak et al., 1999; Spillantini et al., 1998). The mechanism by which aSyn incites pathogenesis is multifarious but commonly proposed to be due to a toxic gain-of-function due to its structural conversion (Lundvig et al., 2005). Why and how this presynaptic protein deposits aberrantly in neurons remain elusive although the NAC region appears pivotal for forming these aggregates (Giasson et al., 2001).

Furthermore, which of the aSyn species are the major culprits in PD is still a matter of debate, with attention shifting from fibrils to soluble amyloid oligomers as key players in the disease process. In fact, a growing number of studies show that oligomers have a higher cytotoxicity compared to the fibrillar form of the proteins (Demuro et al., 2005; Fink, 2006; Kayed et al., 2003; Lambert et al., 1998; Roher et al., 1996; Volles and Lansbury, 2003), suggesting that soluble amyloid oligomers may be the cause of cellular toxicity instead of the fibrillar aggregates (Bemporad and Chiti, 2012; Caughey and Lansbury, 2003; Danzer et al., 2007; Ding et al., 2002; Diógenes et al., 2012; Fink, 2006; Goldberg and Lansbury, 2000; Haass and Selkoe, 2007; Lashuel et al., 2002a; Volles and Lansbury, 2003; Winner et al., 2011). Some studies indicate that fibrillar aggregates

could simply be a storage mechanism and or even be protective (Quist et al., 2005).

Nevertheless, despite the intensive efforts to characterize aSyn is role in PD and its potential as a target for neuroprotective therapies, there are still more questions than answers as to whether and how aSyn plays a direct causative role in PD.

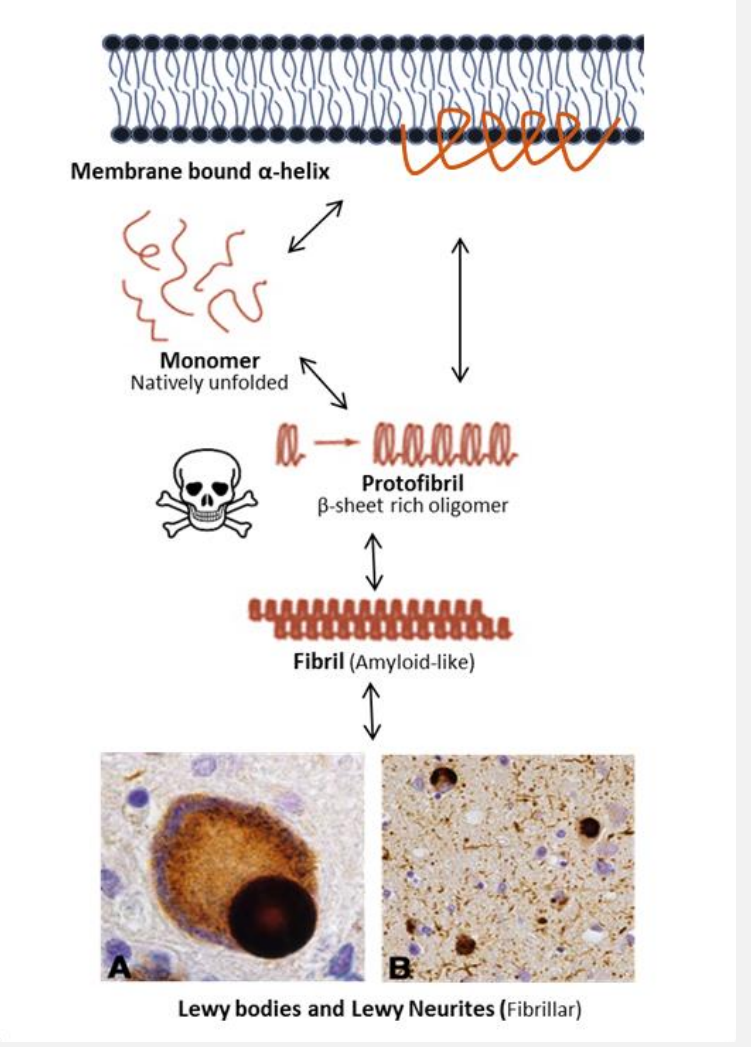


Figure 1.6. A simplified representation of the protein aggregation process. Monomers can self-associate and form fibrillar structures via intermediate oligomeric aggregates (A and B from Takahashi and Wakabayashi, 2005).

Current Concepts on Parkinson's Disease Pathogenic Mechanisms

All the epidemiological findings, pathological observations and genetics discoveries already described above, resulted in substantial advances in the understanding of the pathophysiology of PD. Nevertheless, the underlying mechanisms and precise molecular players involved in neuronal dysfunction and degeneration remain unclear. Numerous and distinct hypothesis concerning cellular imbalances have been implicated in PD pathogenesis including post-translational modifications, impaired protein degradation machinery, formation of pore-like structures with perforation of membranes, mitochondrial malfunction, bioenergetics, dopamine synthesis, Golgi apparatus and transport, as well as oxidative stress, chronic endoplasmic reticulum (ER) stress, transcriptional deregulation, glutamate receptor dysfunction, and neuroinflammation (Dauer and Przedborski, 2003; Kalia et al., 2013; Wales et al., 2013). Below are discussed some of the main hypothesis. Note that the molecular events here mentioned are not mutually exclusive, and some of them can even co-operate to induce PD pathology.

Prion-disease-like mechanisms. PD is a protein-misfolding disorder, sharing fundamental biological properties with other neurodegenerative diseases, including AD, HD, motor neuron diseases and prion diseases. In fact, a particular focus of research suggests a link between prion proteins and small protein aggregates or assemblies - namely oligomers, ribbons, and fibrils - in neurodegenerative diseases. Laboratory studies have demonstrated that small aSyn aggregates may not only cause neuronal death at the site of expression and formation but, may also propagate the neurogenerative process *via* a "prion-like" spreading. Prions are composed of the scrapie prion protein (PrP^{sc}), a misfolded form of the endogenous

cellular PrP (PrP^C), and underlie disorders such as Creutzfeldt-Jakob disease, bovine spongiform encephalopathy, and scrapie (Prusiner, 1998). The conversion of alpha helical PrP^C to the β-sheet rich PrP^{sc} confers an infectivity that is the defining feature of a prion (Pan et al., 1993). Though the precise molecular mechanisms underlying the propagation of the prion protein are unknown, it is universally accepted that PrP^{sc} acts as a template upon which native PrP^C is refolded into PrP^{sc}. Based on this idea, the question whether aSyn-pathology arise as a function of a "prion-like" mechanism, arouse (Costanzo and Zurzolo, 2013; Marques and Outeiro, 2012). This hypothesis was brought forward when *post-mortem* autopsy of a PD patient brain tissue revealed that engrafted nigral neurons also develop LB pathology, suggesting that the healthy transplanted tissue could be transformed by the host tissue (Kordower et al., 2008; Li et al., 2008). Animal studies have also shown the spread of aSyn from the periphery to the brain, after intramuscular, gastric or intravenous injection (Peelaerts et al., 2015; Reichmann et al., 2016). In addition, pathological studies on PD patients suggest that PD pathology progresses from the ENS and the olfactory bulb into the CNS. Preliminary evidence that these findings are relevant to PD has come from epidemiological studies demonstrating a lower risk of developing PD in people who underwent truncal vagotomy (Liu et al., 2017a; Svensson et al., 2015). These findings do not directly demonstrate the spread of aSyn but suggest that an intact *vagus* nerve, which may act as a conduit from the gut to the brain, increases the risk of PD. The current and disputed hypothesis of a "prion-like" dissemination of aSyn is now being studied by many PD research groups and suggests that the spreading of aSyn pathology is due to the cell-to-cell transmission of a pathogenic form of aSyn. This process involves: (1) the release of aSyn from a cell; (2) aSyn uptake by neighboring nerve cells; and (3) subsequently induction of LB pathology in the recipient cell, probably caused by a seeding and self-propagation effect of the

endogenous aSyn, promoting oligomerization and fibrillization of surrounding monomeric aSyn (Costanzo and Zurzolo, 2013).

Membrane disruption and pore formation. One conceivable and vastly studied pathway to cell-to-cell transmission and consequent disruption of cellular homeostasis and toxicity is the permeabilization of cellular membranes (Gallegos et al., 2015; Iljina et al., 2016; Stöckl et al., 2013). It is known that misfolded amyloid proteins may form pore-like structures and elicit channel activity in the membrane (Dobson, 2003). Amyloid ion channels allow ionic exchange across the plasma membrane and thus disrupt the cellular ionic homeostasis. Such ionic exchange ultimately leads to cellular Ca^{2+} loading, which is a common property of amyloidogenic cellular pathophysiology and neurodegeneration and will be further discussed. In accordance, several studies by different groups have demonstrated that one of the pathological properties of aSyn is due to the ability of its oligomers to form pores which change membrane permeability (Tsigelny et al. 2012). The first reports on membrane permeabilization by pore-like annular shaped aSyn oligomers came in the early 2000s by Lansbury group (Lashuel et al., 2002b; Volles and Lansbury, 2002). Many studies came after, showing that aSyn oligomeric species are able to interact and interfere with lipid cellular membranes, increasing membrane conductance and forming ion permeable pore-like structures (Feng et al., 2010; Kim et al., 2009; Kostka et al., 2008; Quist et al., 2005; van Rooijen et al., 2010, 2010; Schmidt et al., 2012; Tsigelny et al., 2008, 2012; Volles and Lansbury, 2002; Zakharov et al., 2007). It has also been reported that mutant A53T and A30P aSyn causes higher membrane permeability and induces the formation of pores in SH-SY5Y cells plasma membrane, which allows Ca^{2+} influx, and therefore plays an important role in cell degeneration (Furukawa et al., 2006). More recently, this hypothesis was tested in neuronal membranes exposed directly to

extracellular aSyn oligomers. Using a variation of the patch-clamp technique, Pacheco and colleagues showed that aSyn oligomers rapidly associate with hippocampal membranes in a punctate fashion and form "pore-like structures", resulting in increased membrane conductance and Ca^{2+} influx (Pacheco et al., 2015). This amyloid pore formation was shown to be dependent on two membrane lipids, ganglioside and cholesterol, that physically interact with amyloid proteins through specific structural motifs (Di Scala et al., 2016a). However, some of these studies were conducted using fairly high concentrations of aSyn, in the µM range, which exceeds largely the physiopathological range of concentration found in cerebrospinal fluid (CSF) of PD patients (Parnetti et al., 2016). Furthermore, another hypothesis suggests that aSyn oligomers to not necessary form pore-like structures but instead, increase membrane permeability by thinning of the hydrophobic core of the lipid bilayer. In this case, the incorporation of the oligomers between tightly packed lipids would facilitate the diffusion of small molecules across the membrane (Bemporad and Chiti, 2012; Stöckl et al., 2013). Plus, the existence of these "pore-like structures" was never observed in living animals.

Calcium dysregulation. Ca^{2+} is the most pleiotropic ion and is able to trigger the majority of intracellular pathways in all cell types in response to external or internal stimuli. In the brain, and especially in neurons, Ca^{2+} plays a fundamental role in synaptic transmission, plasticity, transport, and neuron-neuron and neuron-glia signaling (Carafoli, 2002). Any alterations of the physiological Ca^{2+} signal in neurons or astrocytes lead to changes in signal transduction and cell death. Ca^{2+} dysregulation has been extensively reported in aSyn models of PD as one of the key factors which trigger neuronal dysfunction and degeneration in PD (Brini et al., 2014; Fedrizzi and Carafoli, 2011). Additionally, there are a number of ways in which aSyn and Ca^{2+} might be linked. On one hand, overexpression of

intracellular aSyn in neuroblastoma cell models has been associated with alterations in basal and depolarising-stimulus-evoked Ca^{2+} signals. Accordingly, several studies have shown an increase in basal intracellular Ca^{2+} levels after exposing neurons or astrocytes to exogenous aSyn oligomers (Danzer et al., 2007), with no changes with monomeric or fibrillar forms, confirming a species-specific effect. However, a recente study by Angelova and colleagues, suggests that the application of the unfolded monomeric form of aSyn can also induce an increase in Ca^{2+} levels but with no changes in channel formation as it was observed for the oligomers. Moreover, only Ca^{2+} increase induced by aSyn oligomers is associated with cell death (Angelova et al., 2016). Overall these findings confirm that aSyn interacts with membranes to affect Ca^{2+} signaling and the oligomeric β-sheet-rich aSyn species ultimately lead to Ca^{2+}-dependent cell death. On the other hand, high levels of intracellular Ca^{2+} can promote the intracellular oligomerization and aggregation of aSyn. Thus, there is a complex loop in which aSyn expression and abnormal aggregation might initially promote Ca^{2+} dysregulation, which might, in turn, promote further aggregation (Follett et al., 2013; Nath et al., 2011). Moreover, epidemiological studies suggest that the use of Ca^{2+} channel blockers are associated with a reduced risk of PD (Noyce et al., 2012). The ability of Ca^{2+} channel blockers to reduce oxidative stress in neurons that are susceptible to death in PD has also been proposed to explain these observations. Indeed, previous studies suggest that *SN* neurons are particularly prone to higher levels of basal mitochondrial oxidative stress due to an increasing in intracellular Ca^{2+} (Surmeier and Schumacker, 2013). Therefore, Ca^{2+} channel blockers might protect neurons by decreasing concentrations of reactive oxygen species (ROS).

Oxidative stress. The maintenance of redox homeostasis is critical for the proper function of redox-sensitive signaling proteins in neuron cells as well as for neuronal survival (Apel and Hirt, 2004; Chinta and Andersen, 2008; Scialò et al., 2017). Oxidative stress is caused by an imbalance in the redox state of the cell, either by overproduction of ROS or by dysfunction of the endogenous antioxidant systems (Dias et al., 2013). The role of oxidative stress is increasingly recognized in neurological and neurodegenerative disorders (Gandhi and Abramov, 2012; Halliwell, 2006), with a large body of evidence from *post-mortem* studies implicating oxidative stress in the pathology of PD (Gaki and Papavassiliou, 2014). In the brain, the primary sites of ROS generation include mitochondria in the neurons and glia. The production of these free radicals has been reported to be exacerbated in PD. This can occur due to neuroinflammation, dopamine degradation, mitochondrial dysfunction, aging, glutathione (GSH) depletion, and high levels of iron or Ca^{2+} (Liu et al., 2017b). *In vitro* studies reported that aSyn oligomers, but not fibrils or monomers, are able to induce oxidative stress, resulting in a decreased level of GSH in neurons and astrocytes. Importantly triplication of aSyn also demonstrated a reduction in GSH in human neurons derived from induced pluripotent stem cells (Deas et al., 2016). However, aSyn was also reported to directly activate GSH peroxidase in order to prevent oxidative stress (Koo et al., 2013). Another more controversial hypothesis, attempting to link oxidative stress to the susceptibility of *SNc* neurons to cell death in PD propose that excessive cytotoxic free radicals result from oxidation of cytosolic dopamine and its metabolites or from an overload of free iron within the *SNc* (Greenamyre and Hastings, 2004; Jellinger, 2013; Sian-Hülsmann et al., 2011). Understanding ROS-related mechanisms in PD progression can provide important insights into possible treatments that alleviate PD symptoms.

Mitochondrial dysfunction. The implication of mitochondrial dysfunction in PD pathology has been shown for a long time, both in PD toxic models (rotenone and MPTP) and, more recently, in familial cases of the disease (Burchell et al., 2010a, 2010b; Schapira et al., 1990; Yan et al., 2013). These studies report that soluble, prefibrillar aSyn oligomers, but not monomeric or fibrillar aSyn, inhibit mitochondrial complex I, promote Ca^{2+}-induced mitochondrial swelling and depolarization, and enhance cytochrome c release (Banerjee et al., 2010; Luth et al., 2014; Reeve et al., 2015; Sherer et al., 2002). Importantly, cells with triplication of aSyn also demonstrated mitochondrial dysfunction (Mak et al., 2011; Sarafian et al., 2013). The role of aSyn in mitophagy, mitochondrial fission/fusion and protein trafficking to this organelle has also been suggested (Gottschalk et al., 2014; Mullin and Schapira, 2013). In addition, activation of mitochondrial permeability transition pore (mPTP), a protein that is known to be involved in mitochondrial swelling and damage, was shown to be associated with neurodegeneration in a transgenic (Tg) human aSyn mouse model (Martin et al., 2014). And, interestingly, impaired mitochondrial function, resulting from exposure to toxins, promoted aSyn aggregation (Betarbet et al., 2000; Song et al., 2004). Thus, it is not clear whether toxic aSyn leads to mitochondrial dysfunction or mitochondrial dysfunction causes the formation of pathological forms of the protein, but a synergistic interaction between aSyn and this organelle is likely to play an important role in neurodegeneration (Nakamura, 2013).

Overall, aSyn oligomers can induce toxic Ca^{2+} signaling, which, in turn, activates enzymatic ROS production and the combination of oxidative stress and Ca^{2+} overload affects mitochondria, which can trigger the cell death cascade. Various neuroprotective strategies have been identified to diminish mitochondrial oxidative stress within dopaminergic neurons.

Neuroinflammation. When microglial cell activation was first characterized in the brain of patients with parkinsonian symptoms almost 30 years ago, the hypothesis that neuroinflammation could be involved in the pathophysiology of PD gain attention (McGeer et al., 1988). Since then, *in vitro*, *in vivo* and *post-mortem* studies suggesting that neuroinflammatory mechanisms contribute to the cascade of events which lead to neuronal death and disease progression, emerged. Consistent with this idea, anti-inflammatory drugs, namely non-steroidal anti-inflammatory drugs, were shown to have a protective effect both in animal models and in epidemiological studies (Bassani et al., 2015; Noyce et al., 2012). Despite neuroinflammatory processes being found to also contribute to many other neurodegenerative disorders, such as AD, Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and progressive supranuclear palsy, neuroinflammation does not seem to be merely a consequence of neurodegeneration.

Available data support the importance of non-cell-autonomous pathological mechanisms occurring within areas of neurodegeneration in PD. Most of these mechanisms are mediated by activated microglia, astrocytes, and peripheral immune cells. Astrocytes and microglia are both involved in clearance of extracellular debris, which might aid in the survival of neurons. Furthermore, activated microglia can release trophic factors, such as brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF), but can also release harmful reactive oxygen and nitrogen species and pro-inflammatory cytokines (Hirsch and Hunot, 2009). This cellular response might eventually lead to dopaminergic cell death and, hence, disease progression. Although much evidence from preclinical studies suggests a deleterious role of immune-associated mechanisms in PD, there is still some scepticism regarding this topic, especially due to the overall inability of animal models that can accurately predict the outcomes of trials that test neuroprotection in

humans (Dragunow, 2008). Thus, the ultimate goal of translating our basic understanding of the neuroinflammatory network into therapeutic interventions is still distant and a more vigorous investigation into immune-associated changes in PD is needed.

Although remarkable insights into the pathogenesis of PD have emerged in recent years, many questions remain unanswered and the relative contributions of the different possible mechanisms of PD-pathology are unknown. Considerable challenges need to be explored in order to reconcile the diverse and sometimes contradictory range of studies. Further studies are needed to examine the factors that control the cell-to-cell transfer of aSyn, the impact of inflammation, oxidative stress, mitochondrial dysfunction, membrane disruption, and Ca^{2+} dysregulation on the accumulation of misfolded proteins, synaptic impairment and neuronal degeneration. Hopefully, insight from future studies will translate into an improved molecular understanding of aSyn pathology and ultimately lead to novel and effective therapeutic strategies that interfere with key steps in the pathogenic mechanism, thereby altering the disease progression.

Therapeutic Avenues in Parkinson's Disease

Currently, the pharmacological and non-pharmacological treatments approved for PD only offer symptomatic relief for patients. As these treatments are not able to arrest or reverse the progression of the disease, PD remains incurable.

Dopaminergic therapies for PD. The motor symptoms can be managed with several different drugs that either boost the levels of DA in the CNS or mimic its effects. In 1967 a revolutionary therapeutic breakthrough was made by George Cotzias with the introduction of high dosage levodopa

therapy, a DA precursor also known as L-Dopa (**Fig. 1.7**; Cotzias et al., 1969; Hornykiewicz, 2010). And despite the advances in PD research, levodopa continues to be the gold standard for the treatment of PD nowadays. It is often the first line treatment for patients over 55 years of age and it is typically administered along with carbidopa, a peripheral decarboxylase inhibitor which blocks peripheral conversion of levodopa to DA, thus allowing dose reduction and also minimizing its peripheral adverse effects. This regime is usually effective for 5 years at delaying PD MS. Yet, chronic levodopa treatment is associated with shortened duration of the effect ("wearing off" phenomenon), the development of motor complications (referred as the *long-term levodopa syndrome*; Barbeau, 1976), including motor fluctuations ("on-off" effects) and dyskinesias, nausea, hallucinations, orthostatic hypotension and sleep disturbances (Schapira, 2005; Schapira et al., 2006), that can themselves be disabling. For patients under 55 years of age, a DA receptor agonist (e.g., pramipexole, ropinirole, rotigotine, apomorphine), is typically the first line treatment (Fahn, 2015; Fahn et al., 2004). These drugs are used as the initial choice for dopaminergic therapy, as they delay the need for levodopa, and also as an add-on therapy to levodopa in patients who develop motor complications, since it allows a reduction in levodopa dose. However, most patients who begin treatment with DA receptor agonists will eventually need to add levodopa within a few years. DA receptor agonists are often most effective in addressing mild-to-moderate MS, and are less prone to induce dyskinesia and motor fluctuations, yet they can cause more behavioral disturbances, such as hallucinations, nausea, postural hypotension, somnolence, compulsive gambling, shopping, eating and hypersexuality (Alonso Cánovas et al., 2014; Constantinescu, 2008; Factor, 2001).

Other commonly prescribed drugs for PD MS reduce DA metabolism via selective monoamine oxidase-B (MAO-B) inhibition (e.g., selegiline, rasagiline, and the recently approved safinamide) or catechol-o-methyl-transferase (COMT) inhibition (e.g., entacapone, tolcapone, opicapone) (**Fig. 1.7**; Choy, 2017; Connolly and Lang, 2014; Rizek et al., 2016).

NMS invariably do not respond to dopaminergic medication and are probably the major current challenge faced in the clinical management of PD. Therefore, attention is now being focused on developing new therapeutic interventions that target not only dopaminergic signaling but also non-dopaminergic pathways.

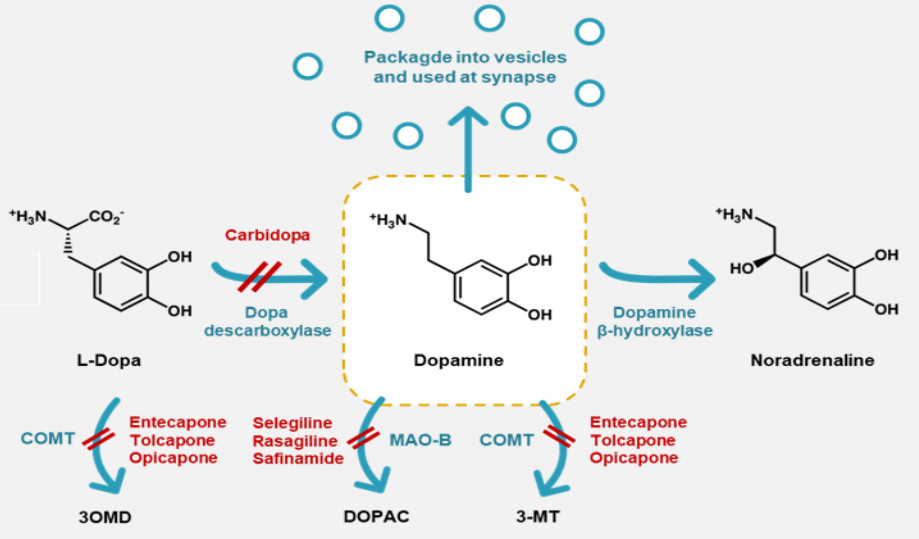


Figure 1.7. Dopamine synthesis and activity in neurons. DA is synthesized from L-aromatic amino acid (L-Dopa or levodopa) by Dopa decarboxylase. DA is then either used, broken down by catechol-O-methyltransferase (COMT) or monoamine oxidase-B (MAO-B) enzymes, or further transformed into noradrenaline by the rate-limiting enzyme dopamine β-hydroxylase. In red, the inhibitors of the corresponding enzymes.

Non-dopaminergic approaches. Non-dopaminergic drug treatments for PD can have two different aims: (1) Targeting motor function. Within the basal ganglia, the main neurotransmission pathways are primarily controlled by gamma-aminobutyric acid (GABA) and glutamate transmission. These pathways have a large range of other neurotransmitter receptors on their surface that are potential targets for drug manipulation of motor function (**Fig. 1.8**). Possible targets include 5-hydroxytryptamine (5-HT; serotonin) receptors, noradrenergic receptors, histamine receptors, nicotinic and muscarinic cholinergic receptors, ionotropic and metabotropic glutamate receptors, and adenosine receptors. (2) Targeting NMS. Outside of the basal ganglia, the pathology of PD affects many other brain *nuclei* (e.g. *locus coeruleus, raphe nuclei,* and dorsal motor nucleus of the *vagus*) and their target structures (e.g. amygdala, hippocampus, and cortex) and a range of neuromodulators and neurotransmitters other than dopamine (e.g. 5-HT, noradrenaline, acetylcholine, GABA, glutamate, and adenosine). These rational provide a focus for using non-dopaminergic drugs to manipulate both the motor and non-motor features of PD.

Non-dopaminergic approaches for the treatment of PD MS have been exploited to a limited degree through the use of amantadine and anticholinergic drugs. Anticholinergics are an alternative and complementary approach to dopaminergic treatments used to restore the balance between cholinergic and dopaminergic inputs on the basal ganglia. Anticholinergic drugs (e.g. trihexyphenidyl, benztropine) are effective for patients with a tremor-predominant phenotype (Singh et al., 2007). The side-effect profile of muscarinic antagonists has limited their use in PD, but there is now interest in pursuing subtype selective M4 antagonists as a means of affecting the basal ganglia without causing cognitive impairment, blurred vision, dry mouth, or urinary retention.

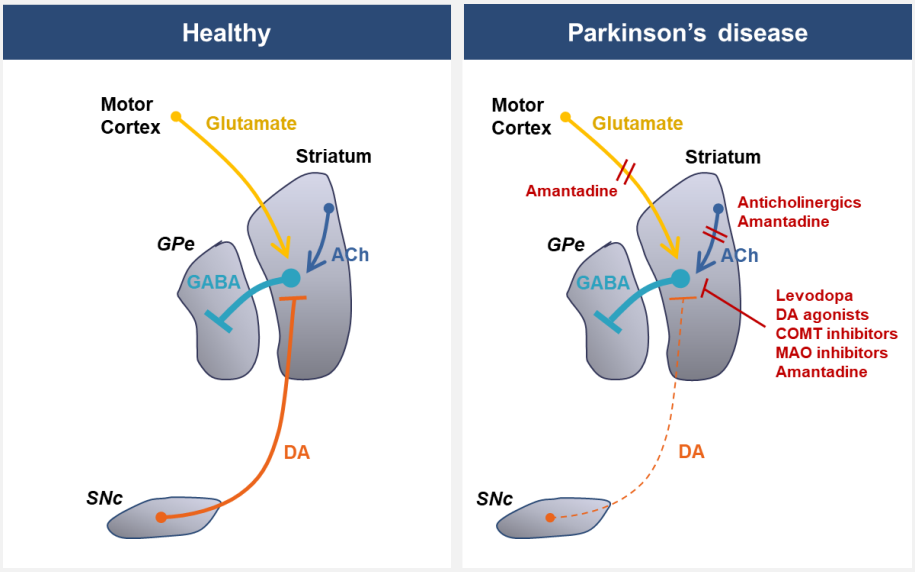


Figure 1.8. Simplified diagram of the neurologic pathways in healthy and PD states, and the major sites of action of drugs used for the treatment of motor features. Dopaminergic afferents from the *SNc*, glutaminergic afferents from the motor cortex, and cholinergic striatal interneurons all converge to exert effects on the activity of GABAergic neurons, which are the main efferent neurons of the striatum. Dopaminergic neurons exert inhibitory effects on GABAergic neurons, whereas cholinergic and glutaminergic neurons produce excitatory effects. The loss of dopaminergic neurons in PD results in a decreased dopamine inhibition, via D_2 receptors, leading to an abnormally high output by GABAergic efferents. This increased inhibitory output causes increased inhibition of the thalamus, as well as reduced excitatory input to the motor cortex.

Amantadine is an anti-influenza agent which was serendipitously found to be useful in PD. Amantadine modulates the release of DA from DA terminals in the striatum, possesses anticholinergic properties and blocks glutamate N-methyl-D-aspartic acid receptor (NMDAR). It may be effective in managing MS in mildly affected patients with early disease and in reducing motor fluctuations in patients with advanced disease (Hubsher et al., 2012). If dyskinesia occurs as PD progresses, amantadine

can also target this particular symptom. However, the use of amantadine has been limited by its side-effects profile, these include insomnia, leg swelling and/or skin discolouration (termed *livedo reticularis*), blurred vision, nausea, dizziness or lightheadedness, and hallucinations.

It is now appreciated that PD is associated with widespread pathology affecting additional neuronal fields and neurotransmitter systems, including the anterior olfactory structures, dorsal motor nucleus of the *vagus*, caudal *raphé nuclei*, *locus coeruleus*, hippocampus, cerebral cortex, and the ANS. Accordingly, continuous studies have shown that the mechanism of PD involves not only dopaminergic but also non-dopaminergic mechanisms involving adenosinergic, cholinergic, adrenergic, serotoninergic, glutamatergic, histaminic and iron chelator pathways (Blandini et al., 1996; Buddhala et al., 2015; Huot et al., 2017; Schiffmann et al., 2007). This widespread pathology is on the basis of PD NMS but, unlike most MS, have often limited treatment options or response (Chaudhuri et al., 2006). Despite the available treatments that can effectively control or improve disability of some of the NMS, namely depression, psychosis, autonomic dysfunction, sleep disorders and fatigue (Kalia and Lang, 2015; Seppi et al., 2011), the current pharmacological therapies for PD cognitive impairments, including dementia, can only ease the symptoms and their benefits are often marginal and non-sustained. These include rivastigmine, donepezil, and galantamine, all acting by increasing cholinergic neurotransmission in the CNS. Additionally, no medications are currently available to treat MCI (Gratwicke et al., 2015; Svenningsson et al., 2012; Szeto and Lewis, 2016). Consequently, there is an urgent need to develop approaches to treatment that are non-dopaminergic in nature.

Adenosine A_{2A} receptor ($A_{2A}R$) antagonists represent a novel and promising class of non-dopaminergic drugs currently under evaluation for the treatment of PD patients. The interest on targeting $A_{2A}R$ in the realm

of PD first arose based on a study using adenosine receptors antagonists in an hemiparkinsonian rat model (Fuxe and Ungerstedt, 1974) and on the potency of methylxanthines, such as caffeine, in enhancing levodopa action (Fredholm et al., 1976). Since then, and based on the ability of purinergic signaling, namely $A_{2A}R$, to fine-tune DA actions in the striatum, $A_{2A}R$ became a focus for treating PD MS. However, the role of central $A_{2A}R$ is now viewed as much broader than just controlling D2 receptor function. Their physiological functions, mechanisms of action, involvement in PD pathology and their potential role as therapeutic targets for both MS and NMS in PD, as well as neuroprotective agents will be further discussed.

Potential neuroprotective agents. Another major therapeutic challenge for PD is the development of disease-modifying treatments to slow or prevent the progression of neurodegeneration. Neuroprotection is proving a difficult issue, with several anti-parkinsonian therapeutics that look highly effective in preclinical animal studies turning out to be ineffective in clinical trials. This has occurred with MAO-B inhibitors, glutamate antagonists, enhancers of mitochondrial function, inhibitors of apoptotic mechanisms, trophic factors, DA agonists, amongst others (Morelli et al., 2009). New approaches are emerging based on epidemiological data, associating several factors with a reduced risk of developing PD. Although some are still controversial, others are already in clinical trials. Examples of these factors are cigarette smoking (with a nicotine patch in phase II trial), the use of nonsteroidal anti-inflammatory drugs, anti-hypertensive agents as Ca^{2+} channel blockers (with isradipine in phase III trial), diabetes (with the anti-diabetic drug exenatide in phase II trial) and caffeine intake (with the adenosine $A_{2A}R$ antagonist istradefylline currently approved in Japan).

Among other agents that are currently being investigated as potential neuroprotective agents are phytochemicals such as isorhynchophylline, resveratrol, trehalose, ginseng, ginkgo biloba, saponins, chronic lithium therapy, cannabis, L-carnitine, estrogen, and NMDAR antagonists (Huang and Adachi, 2016; Mythri et al., 2012; Singh et al., 2007; Sun et al., 2015). Some features that make them good drug candidates are their low cytotoxicity. Since oxidative stress is believed to be involved in the pathogenesis of PD, several compounds with proven antioxidant properties, namely melatonin, selenium, uric acid, vitamins A, C, and E and Coenzyme Q10 (CoQ10) are now being investigated as potential neuroprotective agents for PD (Sarkar et al., 2016; Yang et al., 2016).

Although numerous theories have been proposed and many promising neuroprotective agents have been tested in the clinical trials, none of them was established as a neuroprotective agent. Thus, further research is necessary to develop such an agent in order to mitigate PD pathology.

Non-pharmacological treatments. Surgical therapies may be considered during the progression of PD, especially if symptoms cannot be adequately controlled by pharmacological agents. Similar to the available PD drugs, surgical options offer only symptomatic benefit. While pallidotomy and thalamotomy can still be done in certain situations, they have largely been replaced by deep brain stimulation (DBS). DBS of the STN and GPi can be more effective at managing MS in patients with advanced, medication-refractory PD (Deuschl et al., 2006). Besides, it provides an additional benefit for tremor, rigidity, and bradykinesia; although gait and balance are unlikely to improve, and cognition may be worsened (Fasano et al., 2012). Newer drug delivery systems for PD are increasingly more focused on site-specific delivery of pharmaceuticals. A non-pharmacological

technology currently on clinical trials is a PD vaccine that showed extremely positive results in an animal model of PD (Masliah et al., 2005). The rationale behind PD vaccine is that immunization with aSyn stimulates the immune system to target the toxic form of aSyn promoting its degradation (Romero-Ramos et al., 2014). Human trials on the proposed vaccine, affilope, are currently in phase I. Another promising non-pharmacological treatment for PD under investigation is gene therapy which corrects genetic abnormalities contributing to disease pathogenesis at the molecular level. This approach involves either the introduction of a replacement allele into cells to compensate for the loss of gene function or the silencing of a dominant mutant allele that is pathologic to cells. Since PD is mainly idiopathic and thus not involving one specific genetic target, PD gene therapy also focuses on pathways of cell repair and neuroprotection. Three main approaches are under discussion: (1) induction of DA production, (2) neuronal protection in the *SNc*, and (3) GABA-mediated inhibition of the STN (Choong et al., 2016; Simonato et al., 2013). The clinical delivery of adeno-associated virus encoding human aromatic L-amino acid decarboxylase (AAV2-hAADC), by gene therapy, is now in phase I of clinical trials. This strategy is based on the loss of the enzyme AADC as the dopaminergic neurons die, resulting in a reduction in the conversion of levodopa into DA. Gene therapy to replace AADC should provide significant clinical benefit to PD patients by potentially reducing the required levodopa dose level and dyskinesias associated with high levodopa administration (Mittermeyer et al., 2012). Another promising approach for PD treatment is the transplantation of DA-producing neurons to replace those degenerated during the disease pathogenesis. Regenerative-medicine aims at developing autologous stem cell-derived therapies, with studies demonstrating that mature cells can be coaxed back into stem cells. The potential source for regenerative medicine is the human pluripotent stem cells (hPSCs) which include

human embryonic stem cells (hESCs), human induced pluripotent stem cells (hiPSCs), human neural cells and mesenchymal cells. After transplantation into humans, the neuronal progenitors will eventually derive into DA neurons and rescue the symptoms underlying dopaminergic neuronal loss (Dhivya and Balachandar, 2017).

Overall, currently available pharmacological and non-pharmacological therapies are unable to arrest, nor reverse the advance of this relentlessly progressive and severely debilitating condition. Furthermore, as NMS are a significant determinant of PD patient's quality of life, it is essential that any intervention in PD pathogenesis to slow the disease targets not only dopaminergic signalling but also non-dopaminergic pathways. Lastly, the discrepancy between encouraging preclinical data and failure to translate the results into therapies is likely to be related primarily to our incomplete understanding of the pathogenic mechanisms underlying PD.

Adenosine A_{2A} Receptors as Targets in Parkinson's Disease

Adenosine Physiology

Adenosine is a naturally occurring purine ribonucleoside (**Fig. 1.9**) with the primary and evolutionary conserved function of maintaining a bioenergetic equilibrium. It is distributed ubiquitously throughout the body and is directly involved in key processes sustaining cellular viability and adaptability, such as energy charge (via ATP/ADP), redox control (via NADH), epigenetic control (via the methyl donor SAH), and formation of DNA and RNA (since adenosine contains adenine as its nucleobase). Moreover, all cells have an intracellular metabolism based on adenosine, using this molecule as a paracrine signal to coordinate physiological processes, especially in excitable tissues as the heart and the brain, acting both as a homeostatic transcellular messenger, and as a neuromodulator. The latter is of particular interest within the CNS, where it is involved in the regulation of local neuronal excitability and in a variety of important synaptic processes and signaling pathways. It is considered a neuromodulator rather than a neurotransmitter since it is not stored nor released in synaptic vesicles, as a classical neurotransmitter, and it does not have direct effects on synaptic transmission, but rather plays an important fine-tuning influence on other neurotransmitters and thus modulating neuronal communication.

Adenosine affects neuronal activity through multiple mechanisms: (1) presynaptically by controlling neurotransmitter release and maintaining the inhibitory tone; (2) postsynaptically by modulating the activity of neurotransmitter receptors and other neuromodulators and hyperpolarizing or depolarizing neurons; and (3) nonsynaptically mainly via regulatory effects on glial cells (Boison et al., 2010; Daré et al., 2007;

Fredholm et al., 2005; Ribeiro and Sebastião, 2010; Sebastião and Ribeiro, 2009).

Because adenosine acts as a fine-tuner of synaptic transmission, it has a key role in the homeostatic control of the nervous system activity. When this homeostasis is disrupted, there is an abnormal neuronal communication that can lead to several pathologies of the CNS. Based on this, adenosine has been studied in a wide range of diseases, namely sleep disorders, epilepsy, panic disorder, anxiety, schizophrenia, AD and PD. A promising therapeutic strategy to reestablish neuronal homeostasis in these pathological conditions, is to modulate neurotransmitter receptors or transporters, via adenosine and adenosine receptors (Lopes et al., 2011).

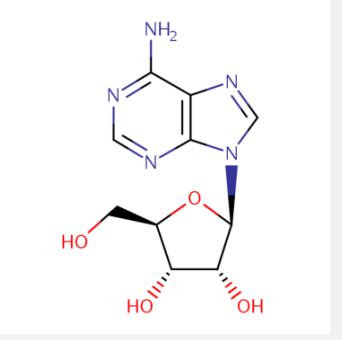


Figure 1.9. Chemical structure of adenosine. Adenosine is a nucleoside, composed of an adenine molecule attached to a ribose sugar molecule (ribofuranose) through a β-N9-glycosidic bond.

Adenosine Synthesis and Metabolism

It is well established that adenosine can be formed in the CNS either intra or extracellularly (**Fig. 1.10**). In the cell, adenosine is an intermediate for the synthesis of nucleic acids and adenosine-5'-triphosphate (ATP), and may be formed through: **(1)** dephosphorylation of cytosolic adenosine triphosphate (ATP), or **(2)** breakdown of adenosine monophosphate

(cAMP) by phosphodiesterase (PDE), both followed by the hydrolysis of 5'-adenosine monophosphate (AMP), catalyzed by cytosolic 5'-nucleotidase. Another intracellular source of adenosine may be **(3)** the hydrolysis of *S*-adenosyl-homocysteine (SAH), by SAH hydrolase (SAHH), an enzyme present in brain areas such as the neocortex, hippocampus, and cerebellum (Latini and Pedata, 2001).

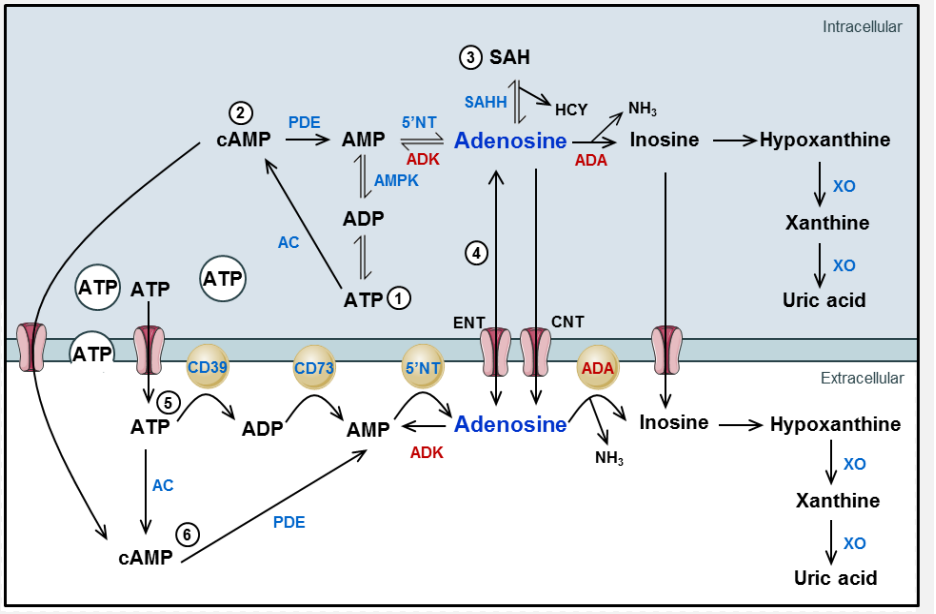


Figure 1.10. Schematic representation of adenosine production, metabolism, and transport. Adenosine can be synthesized intra and extracellularly by nucleotidase enzymes activity. Bidirectional specific transporters, namely equilibrative nucleoside transporters (ENT) or concentrative nucleoside transporters (CNT) regulate the extracellular concentration of adenosine. Abbreviations are as follow: AMP, 5'-adenosine monophosphate; SAH, *S*-adenosyl-homocysteine; SAHH, *S*-adenosyl-homocysteine hydrolase; AC, adenylate cyclase; XO, xanthine oxidase; 5'NT, 5'-nucleotidase; ADA, adenosine deaminase; ADK, adenosine kinase; AMPK, AMP-activated protein kinase; HCY, homocysteine; CD39, cluster of differentiation 39; CD73, cluster of differentiation73; PDE, phosphodiesterase.

The synthesized intracellular adenosine can then (**4**) be transported, as such, to the extracellular medium by facilitated diffusion through bidirectional nucleoside transporters (NT). This can occur after an increase in the intracellular levels of adenosine (e.g., during ischemia, hypoxia, and seizures) or a reversal of sodium gradient and allows the regulation of adenosine extracellular concentrations. There are two main categories of nucleoside transporters: the equilibrative NT (ENT), which carry both purine and pyrimidine nucleosides across cell membranes in both directions and following their concentration gradient; and the concentrative NT (CNT), that mediate influx of nucleosides via the transmembrane sodium gradient. In the CNS the ENT appear to dominate (Thorn and Jarvis, 1996; Young et al., 2013) while CNT, despite operating at the blood brain barrier, are only found at low levels in the brain. Extracellular adenosine can also appear through **(5)** the rapid extracellular conversion of locally released adenine nucleotides (especially ATP) to ADP and then AMP, via the ectonucleotidase pathway (Zimmermann and Braun, 1999), and **(6)** the breakdown of cAMP and direct formation of AMP, via ecto-PDE pathway (**Fig. 1.10**). Adenosine is then formed from AMP by the action of ecto-5'-nucleotidase CD73, a glycolipid-anchored protein thought to be the only relevant enzyme which generates extracellular adenosine in the brain (Lovatt et al., 2012). The pathways (4) and (5) above are the predominant ones and the relative importance of each of them depends on the metabolic state of the cells and, in the case of neuronal cells, their excitability status. Thus, high-frequency neuronal firing and astrocytic stimulation lead to the predominance of the pathway (5) for extracellular adenosine formation (Dunwiddie and Masino, 2001; Fredholm et al., 2005; Latini and Pedata, 2001). Furthermore, the formation of adenosine is dependent on the availability of oxygen and energetic compounds as well as on the rate of synthesis and degradation of ATP, released from both neuronal and glial cells. In fact, it is the release

of ATP from astrocytes, either vesicular (Pascual et al., 2005) or via secretion through hemichannels, that is the major source of synaptic adenosine (Kang et al., 2008; Kawamura et al., 2010). Because ATP is continuously released as a neurotransmitter in the brain, adenosine is continuously produced within the extracellular space. This process of extracellular adenosine formation is very fast and occurs within seconds (Dunwiddie et al., 1997). In basal conditions, adenosine concentrations are normally kept in the range of 30-300 nM (Dunwiddie et al., 1997), by a steady-state expression of adenosine kinase (ADK). At these concentrations, adenosine exerts a tonic inhibitory effect on synaptic transmission.

The bioavailability of adenosine is thought to oscillate with neuronal activity and depends upon its metabolization. Extracellular adenosine is primarily inactivated by uptake across the neuronal cell membrane, followed by either intracellular phosphorylation to AMP by ADK or, to a lesser degree, irreversible deamination to inosine, by adenosine deaminase (ADA). Inosine can then be degraded to hypoxanthine, xanthine, and ultimately uric acid by xanthine oxidase (XO) (Morelli et al., 2010). ADK is a part of the cycle between adenosine and AMP, which sustains the concentration gradient that allows adenosine uptake into cells by ENT. However, conditions prompting an increase in intracellular adenosine will reverse transport direction, leading to release of adenosine into the extracellular space (Gu et al., 1995). Once present in the extracellular space, adenosine may diffuse far away and influence its receptors (Abbracchio and Burnstock, 1998; Abbracchio et al., 2009; Burnstock et al., 2011; Fredholm et al., 2001, 2011; Ribeiro et al., 2002).

Adenosine Receptors and Signaling Pathways

Currently, four subtypes of adenosine receptors have been cloned and characterized: the high affinity A_1R and $A_{2A}R$ (Ka values of 70 and 150 nM, respectively), and $A_{2B}R$ and A_3R which exhibit a lower affinity for adenosine (Ka values of 5100 and 6500 nM, respectively; Dunwiddie and Masino, 2001; Ralevic and Burnstock, 1998; Tucker and Linden, 1993). Interestingly, human A_3R show higher affinity for adenosine but have a low density in most tissues (Fredholm et al., 2001; Ribeiro et al., 2002).

Adenosine receptors are all metabotropic G-protein coupled receptors (GPCR). Classically A_1R and A_3R are coupled to AC inhibitory G proteins (G_i/G_o), whereas $A_{2A}R$ and $A_{2B}R$ are coupled to AC excitatory G proteins (G_s/G_{olf}) (**Fig.1.11**; Fredholm et al., 2001). To note that all adenosine receptors are pleiotropic receptors, meaning that they can potentially couple to different G proteins and to different transducing systems, according to their degree of activation and their particular cellular and subcellular localization (Cunha, 2005). The main adenosine-mediated intracellular signaling pathway involves the formation of cAMP, with A_1R and A_3R stimulation causing, via G_i and G_o proteins, AC inhibition, which leads to reduced protein kinase A (PKA) activity and cAMP response element binding protein (CREB) phosphorylation. On the other hand, stimulation of $A_{2A}R$ and $A_{2B}R$ activates AC, via G_s/G_{olf} proteins, resulting in the activation of PKA, which in turn phosphorylates CREB. However, $A_{2A}R$ have also been reported to couple to G_q proteins, resulting in protein kinase C (PKC) activation (Lopes et al., 1999a). Having adenosine receptors, namely A_1R and $A_{2A}R$, opposite effects, the question how the cell senses the need of the system and decide which receptor should preferentially be activated, arises. Data suggests that at lower frequencies stimulation

and thus low extracellular adenosine concentrations, inhibitory A_1R are predominantly activated. On the contrary, at higher frequencies stimulation and high levels of extracellular adenosine, excitatory $A_{2A}R$ are preferentially activated (Correia-de-Sá and Ribeiro, 1996).

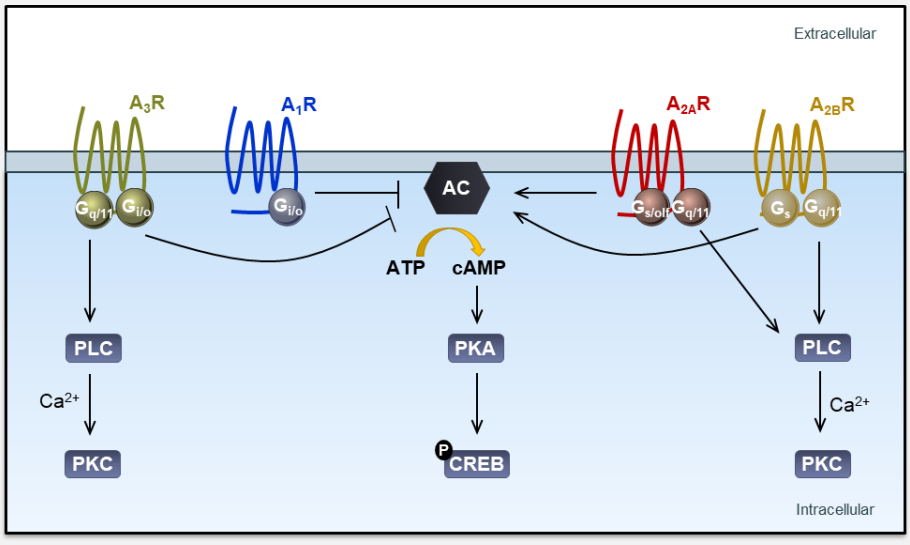


Figure 1.11. Simplified schematic representation of G-protein coupled adenosine receptors. Adenosine A_1R and A_3R are usually coupled to $G_{i/0}$ proteins, decreasing the activity of adenylate cyclase (AC). $A_{2A}R$ and $A_{2B}R$ receptors are usually coupled to $G_{s/olf}$, activating AC and consequently PKA, which in turn phosphorylates CREB. Additionally, A_3R, $A_{2B}R$ and $A_{2A}R$ may couple to $G_{q/11}$ proteins, activating the PLC pathway. Only selected mechanisms and pathways are shown.

Another crucial point for understanding adenosine's differential modulation in the brain, is the distribution of adenosine receptors. In the brain, the adenosine high-affinity receptors, A_1R and $A_{2A}R$, represent the most relevant receptors for adenosine-meditated effects (Fredholm et al., 2005). Adenosine A_1R are the most highly conserved receptor subtype between species, most abundant (it is the second most abundant

metabotropic receptor in the brain), and widely distributed, being highly expressed in cortex, cerebellum, and hippocampus (Reppert et al., 1991). Adenosine $A_{2A}R$, although also widespread in the brain, display high expression levels in neurons of the dorsal and ventral striatum (GABAergic striatopallidal medium spiny neurons and cholinergic interneurons), nucleus *accumbens* in the basal forebrain, and olfactory tubercle (Jarvis and Williams, 1989; Rosin et al., 1998). $A_{2A}R$ are also found in the neocortex and hippocampus albeit with a considerably lower density (Cunha et al., 1994a; Kirk and Richardson, 1995; Sebastião and Ribeiro, 1996). $A_{2A}R$ are thought to play an essential role in the regulation of locomotion, sleep, anxiety, memory, and cognition. $A_{2B}R$ are also widely distributed in the brain but at low levels (Feoktistov and Biaggioni, 1997), and, because they are low affinity receptors, functional studies suggest that they may only be relevant in pathological conditions, when the extracellular adenosine levels are increased. The A_3R are expressed at an intermediate level in the human cerebellum and hippocampus and at lower levels in other brain regions (Dixon et al., 1996; Fredholm et al., 2000; Ribeiro et al., 2002).

Both A_1R and $A_{2A}R$ are mostly located at synapses, in particular glutamatergic excitatory synapses, although both receptors are also found in GABAergic, cholinergic, dopaminergic, serotoninergic and noradrenergic synapses (Cunha, 2016). In the hippocampus, the effects of adenosine under physiological conditions are mostly mediated by A_1R activation and consequent inhibition of glutamate release. Adenosine has, therefore, a tonic inhibitory effect on hippocampal synaptic transmission (Sebastião et al., 1990). Interestingly, despite the low expression and density of $A_{2A}R$ in the hippocampus, these receptors play an important role in the modulation of synaptic transmission. Different effects resulting from $A_{2A}R$ activation were observed, in both neurons and astrocytes (Sebastião and Ribeiro, 2009). The first evidence of $A_{2A}R$ mediated effects on the

hippocampus revealed a presynaptic modulatory effect of $A_{2A}R$ upon A_1R inhibitory actions, resulting in a facilitatory effect on synaptic transmission (Cunha et al., 1994b; O'Kane and Stone, 1998). In neurons, other presynaptic effects were observed later in the modulation of release or uptake of different neurotransmitters, such as glutamate (Lopes et al., 2002), GABA (Cristóvão-Ferreira et al., 2009; Cunha and Ribeiro, 2000) or acetylcholine (Cunha et al., 1994b). Postsynaptically, $A_{2A}R$ are implicated in the modulation of AMPA mediated currents (Dias et al., 2012), and also have been shown to colocalize with mGluR5 and mediate NMDAR signaling (Sarantis et al., 2015; Tebano et al., 2005). Additionaly, postsynaptic activation of $A_{2A}R$ was also implicated in the downstream activation of CREB in the hippocampus (Li et al., 2015).

Evidence also expand $A_{2A}R$ modulatory actions to growth factors and neuropeptides (Cunha-Reis et al., 2007; Diógenes et al., 2004; Flajolet et al., 2008; Gomes et al., 2009; Sebastião et al., 2000). In particular, a crosstalk between $A_{2A}R$ and the neurotrophin receptor TrkB was been described, with important implications for BDNF effects (Assaife-Lopes et al., 2014; Diógenes et al., 2004, 2007; Tebano et al., 2008). $A_{2A}R$ are also found in astrocytes and microglia, where they control Na^+/K^+-ATPase, glutamate release (Nishizaki et al., 2002), and GABA uptake (Cristóvão-Ferreira et al., 2013), as well as the production of pro-inflammatory cytokines. All these effects potentially contribute to the selective $A_{2A}R$-mediated control of synaptic plasticity (Cunha, 2016).

The Role of Adenosine and Adenosine Receptors in Pathology

Brain insults can trigger a large outflow of adenosine and ATP, as a danger signal. Under pathological conditions as ischemia, epilepsy, hypoxia, and excitotoxicity, and also during aging, there is a decrease in the energy charge which leads to an increase of intracellular levels of adenosine that

can than diffuse out of the cell. In these conditions, extracellular adenosine concentrations can rise as much as 10-fold (Dale and Frenguelli, 2009; Fredholm et al., 2000; Latini and Pedata, 2001; Wei et al., 2011) and activate A_1R, which work as a hurdle for damage initiation and signal the noxious stimuli to neighboring cells (Cunha, 2005). A_1R play a key role in neuroprotection by decreasing glutamate release and hyperpolarizing neurons. In fact, studies show that the A_1R activation at the onset of neuronal injury, attenuates brain damage, whereas its blockade exacerbates damage in adult animals.

Interestingly, although adenosine is involved in relevant physiological roles and neuroprotection, it may also contribute to neuronal damage and cell death. In chronic pathological conditions, elevated levels of adenosine will preferentially act on $A_{2A}R$ rather than on A_1R. Consequently, there will be a reduction in the A_1R-mediated inhibition of neurotransmitter release and synaptic transmission (Fernandez et al., 1996) and an increase in the $A_{2A}R$-mediated facilitation of neurotransmission (Fredholm, 1997) which will lead to an adenosinergic inhibitory tonus and the appearance of a stimulatory one. This chronic state where $A_{2A}R$ are preferentially activated can lead to an alteration in the levels of adenosine receptors. In fact, several studies suggest both changes in adenosine and adenosine receptors expression levels in different chronic noxious brain conditions and upon aging. In these situations there is an increase in basal adenosine levels and a down-regulation of A_1R signaling and expression in the cerebral cortex, hippocampus and cerebellum (Cheng et al., 2000; Cunha et al., 1995; Pagonopoulou and Angelatou, 1992; Sebastião and Ribeiro, 2000), and G-protein coupling of A_1R are increased (Cunha, 2001). On the contrary, $A_{2A}R$ expression levels are increased, namely in the cortex and hippocampus (Cunha et al., 1995). Changes in adenosine receptor levels not only impact on the overall effects of adenosine but may also have consequences on the signaling pathways operated by these

receptors. The actions mediated by the activation of adenosine receptors are related to the capacity to modulate neurotransmitters release, synaptic transmission, and plasticity, as well as, to modulate other receptors and molecules, such as neurotrophins, DA receptors, cannabinoid receptors, NMDAR, and mGluR5 receptors (Ferré and Sebastião, 2016; Ribeiro and Sebastião, 2010; Sarantis et al., 2015; Sebastião and Ribeiro, 2009; Tebano et al., 2005, 2008). Moreover, the unbalance of A_1R and $A_{2A}R$ levels has been associated with changes in the transduction mechanisms associated with these receptors (Lopes et al., 1999b). In aged rats, $A_{2A}R$ dependent activation of glutamate release becomes more pronounced and shifts from a PKC mediated signaling to PKA/cAMP/CREB dependent effects (Li et al., 2015; Lopes et al., 1999b, 2002; Rebola et al., 2003), leading to intracellular Ca^{2+} increase and hippocampal-dependent cognitive deficits (Batalha et al., 2016). In young adult animals this effect is dependent on A_1R activation, however, when A_1R are blocked in old animals, no changes are observed, revealing that these effects are no longer being mediated by A_1R (Lopes et al., 1999b). Conversely, $A_{2A}R$ blockade, either with caffeine or selective $A_{2A}R$ antagonists (SCH 58261, KW-6002 or MSX-3), affords neuroprotection in models of epilepsy, depression, AD and PD (Arendash et al., 2006; Cunha et al., 2008; Dall'Igna et al., 2007; Laurent et al., 2014; Viana da Silva et al., 2016), prevents hippocampal-dependent memory deficits and LTP impairments in aged animals (Costa et al., 2008; Prediger et al., 2005), and markedly reduce cerebral ischemic damage in animal models of focal and global ischemia. Furthermore, knocking-out $A_{2A}R$ rescues stress and AD-related synaptic dysfunction (Kaster et al., 2015; Laurent et al., 2014).

Therapeutic Potential of $A_{2A}R$ in Parkinson's Disease

The interest in de adenosine system mostly stems from the recognition that its main function is to assist in maintaining homeostasis in biological systems. In pathological conditions, this adenosinergic system is often impaired. Hence, it can be considered a system of choice to manipulate brain circuits in order to restore their proper function. As mentioned before, several studies show an overactivation and overexpression of $A_{2A}R$ in aging and in different deleterious brain conditions such as PD, as so, the blockade of these receptors has been proposed to be therapeutically useful in these situations (Cunha et al., 1995; Diógenes et al., 2007; Lopes et al., 1999a). Moreover, epidemiological studies show an inverse relation between the consumption of caffeine and the risk of developing PD and memory disruption during aging, AD and PD (Arendash and Cao, 2010; Ascherio et al., 2001; Eskelinen et al., 2009; Flaten et al., 2014; Maia and de Mendonça, 2002; Ritchie et al., 2007). More recently, polymorphisms in the human $A_{2A}R$ gene (ADORA2A) have been linked to a reduced risk of PD (Popat et al., 2011). As such, adenosine $A_{2A}R$ emerged as a potential target for the treatment of PD, and several lines of research focusing on the different properties and roles of $A_{2A}R$ arose, namely: (1) the role of $A_{2A}R$ in the control of neurodegeneration in several noxious brain conditions and neurodegenerative disorders; (2) their role in controlling dopamine D_2 signaling and consequently motor function; and (3) the $A_{2A}R$ effects on memory and cognition. These three lines of evidence are discussed below.

$A_{2A}R$ and neuroprotection. One of the major limitations of the current pharmacological treatment of PD is represented by its substantial ineffectiveness in halting neuronal degeneration. According to the classical view of neuronal damage as resulting from glutamate excitotoxicity (Mattson, 2003), it has been emphasized that the blockade

of $A_{2A}R$ may potentially represent a valuable approach in counteracting neurodegeneration in PD. In fact, $A_{2A}R$ pharmacological blockade or genetic deletion has been associated with neuroprotective effects in several animal models of PD, namely toxin-induced models, MPTP, 6-hydroxydopamine (6-OHDA), and rotenone models (Bové et al., 2005; Chen et al., 2001a; Ikeda et al., 2002; Pierri et al., 2005; Schwarzschild et al., 2003; de Souza et al., 2017). These studies demonstrate that $A_{2A}R$ is involved in the degeneration of the dopaminergic neurons projecting from *SN* onto the striatum. In alignment, $A_{2A}R$ have also been proposed to play an instrumental role in aSyn-mediated neurodegeneration. In this study, aSyn-induced damage to striatal neurons was clearly reduced in $A_{2A}R$ KO mice (Kachroo and Schwarzschild, 2012).

Despite the neuroprotection elicited by $A_{2A}R$ antagonists clearly manifested in different PD animal models, the neuronal mechanism underlying this effect, or the impact to other brain areas, namely cognitive associated areas, have not yet been ascertained. Possible mechanisms underlying neuroprotection include the normalization of glutamatergic synapses by $A_{2A}R$ blockade. This is due to a combined ability of $A_{2A}R$ to facilitate the release of glutamate and the activation of NMDAR. Furthermore, $A_{2A}R$ also control glia function and brain metabolic adaptation, two other emerging mechanisms to understand PD pathogenesis. In addition, neuroprotection also depends on the ability of $A_{2A}R$ to control mitochondria-induced apoptosis. Thus, $A_{2A}R$ might not only control the trigger of neuronal dysfunction of brain circuits (e.g. glutamate excitotoxicity) but also their main system of amplification (e.g. neuroinflammation and metabolic imbalance) as well as their main effector system (e.g. apoptotic-induced neuronal damage). Therefore, $A_{2A}R$ are now conceived as a normalizing device promoting adequate adaptive responses in neuronal circuits. This makes $A_{2A}R$ particularly attractive to

manage early synaptic dysfunction in PD and cognitive-associated deficits, considered one of the most disabling symptoms of PD.

To date, no clinical studies have demonstrate $A_{2A}R$ neuroprotective effects. However, as previously mentioned, epidemiological studies consistently show a lower risk of developing PD in individuals that regularly consume caffeine throughout their lifetime (Ascherio et al., 2001). A randomized controlled trial also demonstrated that treating PD patients with caffeine improved objective motor measures (Postuma et al., 2012). Beneficial effects prompted by caffeine have been largely attributed to $A_{2A}R$ antagonism at dosages equivalent to 3-5 mg/kg.

In sum, direct evidence of neuroprotection mediated by $A_{2A}R$ antagonist in experimental animal models, as well as data from epidemiological studies, provide new insights into the study of the antiparkinsonian potential of the drugs. Yet, the ability of $A_{2A}R$ to impact on brain tissue damage is still a matter of a hot debate and whether $A_{2A}R$ antagonists can be considered as neuroprotective or not, still needs further clarification and a direct evidence in humans.

$A_{2A}R$ as targets for PD motor symptoms. Probably the most exhaustive documentation of the use of $A_{2A}R$ antagonists in PD, namely in PD motor symptoms, was prompted by the particularly high abundance of $A_{2A}R$ in the basal ganglia (Fredholm et al., 2005; Schiffmann et al., 2007). In this particular case, targeting adenosine $A_{2A}R$ first arose based on its tight physical and functional interaction with dopamine D_2 receptors in the striatum. In fact, one of the main actions of $A_{2A}R$ is the control of dopaminergic signaling, which plays a key role in striatal signal processing and thus in motor control (Schiffmann et al., 2007). $A_{2A}R$ and D_2 receptors have opposing effects on cAMP production in cells, such that activation of $A_{2A}R$ inhibits dopamine D_2 receptor signaling. Conversely adenosine $A_{2A}R$ antagonists have been shown to enhance D_2 dependent signalling

(Shook and Jackson, 2011). These drugs provide benefit by inhibiting the overactive striatopallidal pathway (Hauser and Schwarzschild, 2005). In accordance, several studies show that $A_{2A}R$ blockade improves motor function in different rodent and primate models of PD (Armentero et al., 2011; Morelli et al., 2009; Pinna, 2014) and tends to attenuate dyskinesia (Cunha, 2016; Xiao et al., 2006). Nevertheless, the role of central $A_{2A}R$ is now viewed as much broader than just controlling D_2 receptor function. It is now clear that $A_{2A}R$ can control motor function in the absence of dopaminergic signaling (Chen et al., 2001b; Shiozaki et al., 1999). This indicates that striatal $A_{2A}R$ work through dopamine-independent mechanisms to impact on brain function. The concept of dopamine-independent effects of $A_{2A}R$ function is particularly relevant in the case of extra-striatal $A_{2A}R$, where dopaminergic signaling is far less intense (Bastia et al., 2005; Huang et al., 2006; Xiao et al., 2006). Studies identified a critical role of $A_{2A}R$ in extra-striatal neurons in providing a prominent excitatory effect on psychomotor activity (Shen et al., 2008). These extra-striatal receptors were shown to be located in glutamatergic synapses, including striatal glutamatergic terminals, and control both the release of glutamate as well as NMDAR (Lopes et al., 2002; Marchi et al., 2002; Rebola et al., 2005a, 2008). Thus, a major role of $A_{2A}R$ is to normalize the functioning of glutamatergic synapses which dysfunction seems a common feature of many chronic brain diseases. This view comes in line with the robust neuroprotection afforded by $A_{2A}R$ blockade previously discussed.

$A_{2A}R$ and cognitive function. Consumption of coffee is well documented to increase alertness and there is a trend to consider that caffeine improves performance and cognition, especially in situations of cognitive impairment (Chen et al., 2007; Cunha, 2005; Sawyer et al., 1982; Takahashi et al., 2008). Several data show that caffeine and selective

adenosine $A_{2A}R$ antagonists can improve memory performance in rodents evaluated through different tasks. The effects of $A_{2A}R$ on memory are further substantiated by the ability of $A_{2A}R$ to impact working memory (Chen, 2014) and reference memory performance (Cunha and Agostinho, 2010). In fact, in experimental models of aging, chronic stress, early stages of Huntington's disease, deficit hyperactivity disorder (ADHD), diabetic encephalopathy, early life convulsions, AD, and PD, pharmacological or genetic blockade of $A_{2A}R$ impedes memory deterioration and can enhance cognitive function (Bara-Jimenez et al., 2003; Batalha et al., 2013; Canas et al., 2009; Dall'Igna et al., 2007; Kaster et al., 2015; Laurent et al., 2016; Li et al., 2015; Lopes et al., 2011; Prediger et al., 2005).

Importantly, this ability of $A_{2A}R$ blockade to improve both motor and cognitive functions in animal models of neurological disorders, was shown to correlate with neuroprotection not only in the *SNc* and striatal areas but also in extra-striatal brain regions. Consequently, $A_{2A}R$ activity in brain may achieve the modulation of cognitive function, particularly those associated with degenerative disorders, through its control of neuronal death in extra-striatal brain areas.

Clinical testing of adenosine $A_{2A}R$-based therapies. In recent years, there has been a wide array of adenosine-based therapies testes in both preclinical and clinical research for multiple diseases. Clinical trials in PD patients showed that the selective $A_{2A}R$ antagonist KW-6002, also known as istradefylline, reduces the OFF time and potentiates motor improvement when co-administered with low doses of L-DOPA (levodopa) (Kadowaki Horita et al., 2013; Kondo et al., 2015; LeWitt et al., 2008; Mizuno et al., 2010; Pinna, 2014; Uchida et al., 2014). The proposed advantage of this strategy is a reduction in the required dose of L-DOPA, with concomitant reductions in the associated side effects, consisting mainly of dyskinesias and progressive cognitive impairment (Armentero et al., 2011). Likewise,

other $A_{2A}R$ antagonists, namely tozadenant and preladenant, also ameliorate the off time and dyskinesia in PD patients under L-DOPA therapy (Factor et al., 2013; Hauser et al., 2014, 2015). **Table 1** outlines examples of recent clinically tested adenosine $A_{2A}R$-based therapies, their mechanisms of action, and the success of the trial.

Although several successes have been reported (Atack et al., 2014; Kadowaki Horita et al., 2013; Kondo et al., 2015; Pinna, 2014; Pugliese et al., 2009; Uchida et al., 2014; Yuzlenko and Kieć-Kononowicz, 2006), many drugs have failed in clinical trials. At this moment, no clinical trial on adenosine-based therapy is ongoing, but istradefylline is already approved in Japan as an adjunctive treatment for PD (Dungo and Deeks, 2013; Kondo et al., 2015; Mizuno et al., 2010; Uchida et al., 2014). Nevertheless, the introduction of $A_{2A}R$ antagonists in clinics as anti-parkinsonian agents is hoped to bolster our knowledge on the role of $A_{2A}R$ in PD pathology, and consequently open new windows to tackle motor dysfunction and, more importantly, cognitive deficits, which represent now one of the major unmet needs in PD.

Table 1. Name, mechanism of action (MOA), use in clinical trials and respective references (Ref) of adenosine $A_{2A}R$ antagonists.

Drug & MOA	Clinical Trial	Result	Ref
Caffeine (non-selective antagonist)	1. Motor manifestations of PD (Phase 2) 2. Study investigating caffeine for excessive daytime somnolence in PD (Phase 2 & 3) 3. Caffeine as a therapy for PD (Phase 3).	1. Completed (NCT01190735) 2. Completed (NCT00459420) 3. Ongoing (NCT01738178)	1
Istradefylline, KW-6002 ($A_{2A}R$ antagonist)	1. Study of Istradefylline for the treatments of PD in patients taking levodopa (Phase 3) 2. Long-term study in PD patients (Phase 3) 3. A 12-week randomized study in subjects with moderate to severe PD (Phase 3) 4. The effects of mild Hepatic impairment on the pharmacokinetics of Istradefylline (Phase 1) 5. An extension of Istradefylline in North American PD patients who have completed study 6002-INT-001 (Phase 3) 6. The effects of rifampin on the metabolism of Istradefylline in healthy volunteers (Phase 1) 7. KW-6002 to Treat PD (Phase 2)	1. Completed (NCT00955526) 2. Completed (NCT00957203) 3. Completed (NCT01968031) 4. Completed (NCT02256033) 5. Terminated (NCT00199381) 6. Completed (NCT02174250) 7. Completed (NCT00006337)	2, 3, 4, 5
Preladenant ($A_{2A}R$ antagonist)	1. A placebo- and active-controlled study in early PD, (Phase 3) 2. a) A placebo- and active-controlled study in subjects with moderate or severe PD (Phase 3) b) An active-controlled extension study to NCT01155466 [P04938] and NCT01227265 [P07037] (Phase 3) 3. A placebo controlled study in patients with moderate to severe PD (Phase 3) 4. A dose finding study for the treatment of PD (Phase 2)	1. Terminated (NCT01155479) 2. a) Completed (NCT01155479) b) Terminated (NCT01215227) 3. Completed (NCT01227265) 4. Completed (NCT01294800)	6, 7
SYN-115 ($A_{2A}R$ antagonist)	1. An fMRI study in cocaine dependent subjects 2. Safety and efficacy study in PD patients using levodopa to treat end of dose wearing off (Phase 2 & 3)	1. Completed (NCT00783276) 2. Completed (NCT01283594)	8, 9, 10
PBF-509 ($A_{2A}R$ antagonist)	1. Study to assess safety and tolerability of PBF-509 single daily oral dose administration in young male and female healthy subjects (Phase 1) 2. Study to assess the safety and tolerability of PBF-509 in male healthy volunteers (Phase 1)	1. Completed (NCT02111330) 2. Completed (NCT01691924)	

1 (Postuma et al., 2012); 2, 3, 4, 5 (Armentero et al., 2011; Dungo and Deeks, 2013; Mizuno et al., 2010; Rauck et al., 2015); 6, 7 (Hattori et al., 2016; Hauser et al., 2015); 8, 9, 10 (Hauser et al., 2014; Lane et al., 2012; Moeller et al., 2012)

Aim

Research conducted in the last two decades provided important advances into molecular players and pathways involved in PD and other synucleinopathies, but still, there is much to understand and discover. The precise mechanisms involved in PD progression, the molecular basis of cognitive deficits, and the pathological role of its major culprit, aSyn, are unclear.

Novel approaches to PD therapy are sought, and adenosine $A_{2A}R$ emerged as a attractive target for the treatment of both motor and non-motor symptoms. However, the precise molecular mechanisms underlying potential neuroprotection and the effects of $A_{2A}R$ on early cognitive impairments driven by aSyn, remain unknown.

In this context, the work described in this thesis aimed to explore the pathological underpinnings of aSyn-associated synaptic dysfunction and neuronal death and gain insight into the novel concept of a crosstalk between aSyn and $A_{2A}R$, specifically focusing on the early stages of the disease and cognitive-associated brain areas. In order to accomplish this general aim, three specific tasks were designed:

I. Explore the ability of $A_{2A}R$ to modulate aSyn-mediated synaptic dysfunction, oligomerization and aggregation, and lastly, neuronal death.

II. To clarify the molecular pathways involved in aSyn-driven toxicity and identify novel key molecular players here involved.

III. To assess whether the *in vivo* pharmacological blockade of $A_{2A}R$ constitutes a valid strategy for treating early synaptic impairments and cognitive deficits, using an aSyn mice model.

Chapter II

Adenosine A_{2A} Receptors Modulate aSyn Aggregation and Toxicity

Diana G. Ferreira designed and performed the experimental work and wrote the manuscript; except for the production of aSyn species and SDS page profiling (Hugo Vicente-Miranda) and qPCR analysis (Rui Gomes).

Abstract

Abnormal accumulation of aggregated alpha-synuclein (aSyn) is a hallmark of sporadic and familial Parkinson's disease (PD) and related synucleinopathies. Recent studies suggest a neuroprotective role of adenosine A_{2A} receptor ($A_{2A}R$) antagonists in PD. Nevertheless, the precise molecular mechanisms underlying this neuroprotection remain unclear.

We assessed the impact of $A_{2A}R$ blockade or genetic deletion ($A_{2A}R$ KO) on synaptic plasticity and neuronal cell death induced by aSyn oligomers. We found that impairment of LTP associated with aSyn exposure was rescued in $A_{2A}R$ KO mice or upon $A_{2A}R$ blockade, through a NMDA receptor-dependent mechanism. The mechanisms underlying these effects were evaluated in SH-SY5Y cells overexpressing aSyn and rat primary cortical cultures exposed to aSyn. Cell death in both conditions was prevented by selective $A_{2A}R$ antagonists. Interestingly, blockade of these receptors did not interfere with aSyn oligomerization, but, instead, reduced the percentage of cells displaying aSyn inclusions.

Altogether, our data raise the possibility that the well-documented effects of $A_{2A}R$ antagonists involve the control of the latter stages of aSyn aggregation, thereby preventing the associated neurotoxicity. These findings suggest that $A_{2A}R$ represent an important target for the development of effective drugs for the treatment of PD and related synucleinopathies.

Keywords: α-Synuclein, adenosine A_{2A} receptors, hippocampus, neuroprotection, Parkinson’s disease.

Introduction

Parkinson's disease (PD) is a progressive and chronic neurodegenerative disorder characterized by the loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNc), which underlies the classical motor symptoms of the disease (Jankovic, 2008). Another important neuropathological hallmark of the disease is the presence of intraneuronal inclusions known as Lewy bodies (LB) and Lewy neurites (LN; Braak et al., 2003). These deposits occur early in the disease process and are accompanied by progressive neuronal dysfunction and, eventually, the death of the afflicted neuronal populations (Braak and Del Tredici, 2008). Behavioral and cognitive deficits are concomitant with these pathological changes (Turner, 2002). In fact, the accumulation of LB and LN is not restricted to the *SN* but is extended to several brain areas including those related to memory, such as the hippocampus and cortex (Braak et al., 2004; Mattila et al., 2000; Spillantini et al., 1997). Moreover, recent *post-mortem* studies in PD patient brains suggest a correlation between cognitive deficits or dementia and the accumulation of cortical LB (Braak et al., 2005; Caviness et al., 2011; Kövari et al., 2003). These inclusions are mainly composed of fibrillar aggregates of aSyn, a neuronal presynaptic protein associated with both familial and sporadic forms of PD (Hamza et al., 2010; Satake et al., 2009; Simón-Sánchez et al., 2009).

Recently, adenosine A_{2A} receptors ($A_{2A}R$) emerged as an attractive non-dopaminergic target for the treatment of motor and non-motor symptoms (MS; NMS) of PD. Anti-parkinsonian actions are achieved through the blockade of this receptor, whose expression and function become aberrant throughout aging and in age-related pathologies, including the early stages of PD (Lopes et al., 1999a; Varani et al., 2010; Villar-Menéndez et al., 2014). This strategy was also proven beneficial in other diseases associated with neuronal dysfunction, such as epilepsy,

acute and chronic stress and Alzheimer's disease (AD; Batalha et al., 2013; Canas et al., 2009; Cunha, 2005; Laurent et al., 2014a). In addition, epidemiological studies show an inverse correlation between the consumption of caffeine, an $A_{2A}R$ antagonist, and the risk of developing PD (Ascherio et al., 2001).

Furthermore, $A_{2A}R$ deregulation was suggested to play an important role in aSyn mediated neurotoxicity, since aSyn induced damage to striatal neurons was clearly reduced in $A_{2A}R$ KO mice (Kachroo and Schwarzschild, 2012). However, the extent to which $A_{2A}R$ are involved in aSyn-associated toxicity, the underlying protective molecular mechanisms, or the impact on other brain areas are still unknown. Therefore, the purpose of this study was to gain insight into the novel concept of a crosstalk between aSyn and $A_{2A}R$ and to explore the ability of $A_{2A}R$ to modulate aSyn-mediated synaptic dysfunction, formation of inclusions, and neuronal death. To test these hypotheses, we first assessed the functional outcomes of the pharmacological blockade or genetic deletion of $A_{2A}R$ on rodent hippocampal slices exposed to extracellular aSyn oligomers. Then, we set out to determine how $A_{2A}R$ affect aSyn-mediated cell death, and more importantly, whether modulation of $A_{2A}R$ function impacts on aSyn aggregation and oligomerization.

Here, we show for the first time that selective adenosine $A_{2A}R$ antagonists rescue both exogenous and endogenous aSyn-associated cell death. In addition, we found that $A_{2A}R$ modulation alters the formation of aSyn inclusions in cultured cells. Furthermore, the toxic effects of aSyn oligomers on synaptic function are fully prevented by $A_{2A}R$ blockade or deletion, through a mechanism dependent on NMDA receptor (NMDAR). These findings suggest that $A_{2A}R$ represent an important target for the development of effective drugs for the treatment of PD and related synucleinopathies.

Results

$A_{2A}R$ blockade rescues neuronal cell death induced by exogenous aSyn oligomers

First, we compared the effect of different aSyn species on rat primary hippocampal cultures. Cells were incubated with either monomeric or oligomeric aSyn preparations for different time periods. The confirmation of the biochemical properties of the different species was performed by SDS-PAGE/immunoblot analysis, revealing that monomers migrated with a typical molecular weight of ~15 kDa (monomeric molecular weight of aSyn) while oligomeric forms migrated with an apparent molecular weight of >30 kDa and <180 kDa (**Fig. 2.1d**). We found no significant cytotoxicity in cells treated with either aSyn species (500 nM) for 90 min, as assessed by PI and Syto-13 staining. Conversely, we found that after 24 h of exposure there was a robust increase in neuronal cell death upon incubation with aSyn oligomers (cell viability $_{CTR}$ = 68.7 ± 4.6%; cell viability $_{aSyn\ olig,\ 24h}$ = 35.5 ± 3.1%; n = 4; P < 0.001; **Fig. 2.1a, b**), while monomeric species had no effect on cell viability.

We next investigated whether adenosine $A_{2A}R$ could attenuate the neurotoxic effects induced by exposure to aSyn oligomers. The blockade of $A_{2A}R$, using the selective antagonists SCH 58261 (50 nM) or ZM 241385 (50 nM), significantly reduced neuronal cell death induced by aSyn oligomers, resulting in levels similar to control (cell viability $_{SCH\ 58261\ +\ aSyn\ olig,\ 24h}$ = 70.6 ± 4.3%; cell viability $_{ZM\ 241385+\ aSyn\ olig,\ 24h}$ = 69.7 ± 3.2%, n = 4; P < 0.001 *vs*. CTR; **Fig. 2.1a, c**).

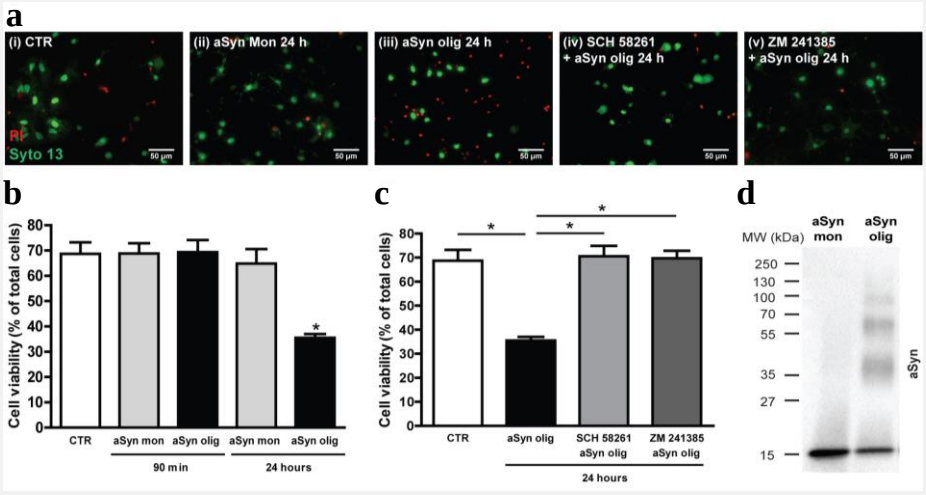


Figure 2.1. Neuronal death induced by extracellular aSyn oligomers is prevented by $A_{2A}R$ blockade. (**a**) Representative images of hippocampal cultures labeled with propidium iodide (PI) and Syto-13 in (i) control conditions (CTR), (ii) after 24 h incubation with aSyn monomers (aSyn mon, 500 nM), (iii) or with aSyn oligomers (aSyn olig, 500 nM) alone or in the presence of the selective $A_{2A}R$ antagonists, (iv) SCH 58261 (50 nM) or (v) ZM 241385 (50 nM). Scale bar: 50 µm. (**b**) Cell viability upon incubation with extracellular aSyn mon or olig. Only 24 h incubation with aSyn olig leads to a decrease in cell viability compared to CTR. (**c**) Rescue of cell viability after exposure to aSyn olig for 24 h by the selective $A_{2A}R$ antagonists, SCH 58261 (50 nM) or ZM 241385 (50 nM). (**d**) SDS-PAGE separation of the different aSyn species (monomers and oligomers). Monomers migrate with monomeric molecular weight (15 kDa) whereas aSyn oligomers display SDS-resistant high molecular weight species. $P < 0.001$. Cell viability is presented as the percentage of living cells compared to the number of total cells counted. All values are mean ± SEM of 4 independent experiments.

Pharmacological or genetic blockade of $A_{2A}R$ prevents LTP impairment induced by aSyn oligomers

We have previously demonstrated that aSyn oligomers, but not monomers or fibers, impair synaptic plasticity and increase basal synaptic transmission through NMDAR activation (Diógenes et al., 2012). Thus, we next set out to investigate the role of $A_{2A}R$ on this impairment of synaptic function. For this, we pre-incubated rat hippocampal slices with aSyn oligomers together with the selective $A_{2A}R$ antagonist SCH 58260 (50 nM, 110 min; **Fig. 2.2a**), and induced LTP in Schaffer-collaterals/CA1 pyramid glutamatergic synapses by theta burst stimulation. The LTP amplitude was significantly reduced in slices pre-incubated with aSyn oligomers (500 nM, 90 min) when compared to control slices (LTP_{CTR} = 58.1 ± 7.4%; $LTP_{aSyn\ olig}$ = 7.7 ± 2.3%; n = 9; $P < 0.001$; **Fig. 2.2b, d**). When $A_{2A}R$ were blocked by SCH 58261, the LTP magnitude was reestablished to control values ($LTP_{SCH\ 58261+\ aSyn\ olig}$ = 50.6 ± 13.0%; n = 5; $P < 0.01$ *vs.* $LTP_{aSyn\ olig}$; **Fig. 2.2b, d**). SCH 58261 alone did not affect LTP magnitude ($LTP_{SCH\ 58261}$ = 52.5 ± 10.6%; n = 5; $P < 0.05$ *vs.* LTP_{CTR}; **Fig. 2.2b, c**).

To assess the role of $A_{2A}R$ on NMDAR mediated effects, we evaluated the effect of the NMDAR antagonist APV (50 μM) on basal synaptic transmission. As expected, APV did not modify the fEPSP slope in control slices (**Fig. 2.2f**). In contrast, the acute application of APV induced a progressive reduction of the fEPSP in aSyn oligomer-treated slices ($fEPSP_{CTR}$ = 100.0 ± 1.2%; $fEPSP_{aSyn\ olig}$ = 87.6 ± 2.1%; n = 6-7; $P < 0.001$; Fig. **2.2e,f**), in agreement with the previously reported impact of oligomeric aSyn on NMDAR (Diógenes et al., 2012). Interestingly, when slices were pre-incubated with SCH 58261 together with aSyn oligomers, the effect of the NMDAR antagonist was prevented ($fEPSP_{SCH\ 58261+\ aSyn\ olig}$ = 94.8 ± 1.0%; n = 4; $P < 0.05$ *vs.* $fEPSP_{aSyn\ olig}$; **Fig. 2.2e, f**).

Accordingly, we observed an increase in NMDAR subunit 2B (NMDAR2B; 137.9 ± 9.4%; n = 3; P < .005 *vs* CTR; **Fig. 2.2h**) in slices exposed to aSyn, but not in NMDAR subunit 1 (NMDAR1). This increase was prevented by co-incubation with SCH 58261 (95.1 ± 11.9%; n = 3; P < .005 *vs* CTR; **Fig. 2.2h**). SCH 58261 alone did not alter NMDAR2B.

In order to evaluate whether A_{2A}R blockade also rescued baseline synaptic efficiency, input/output (I/O) curves were recorded. Slices pre-incubated with aSyn oligomers alone showed a shift to the left in the I/O curve, as previously described (Diógenes et al., 2012). SCH 58261 co-incubation did not change this aSyn-induced effect (**Fig. 2.2g**).

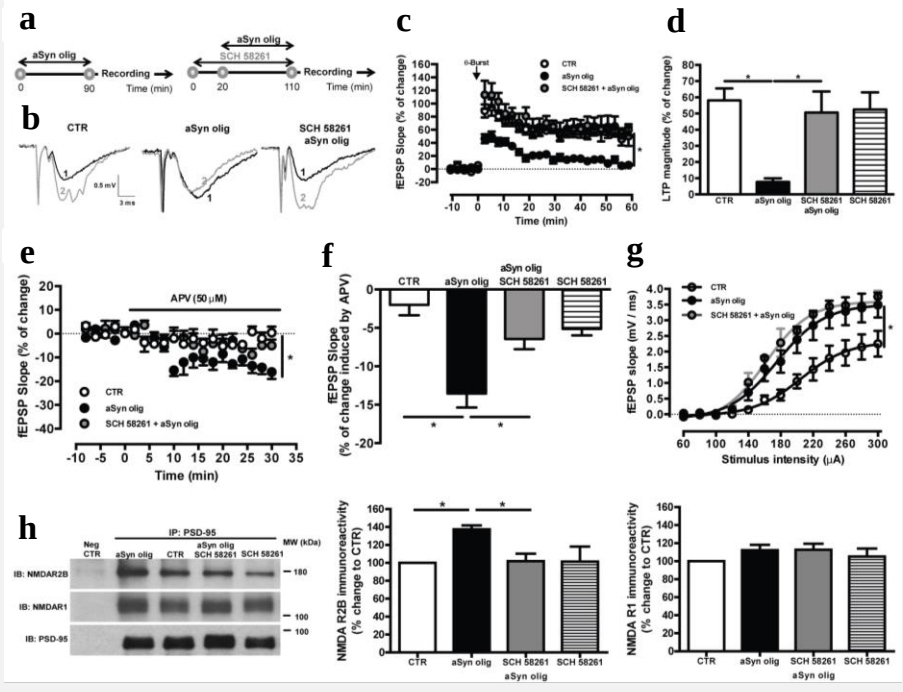


Figure 2.2. A_{2A}R blockade rescues LTP impairment induced by extracellular aSyn oligomers. (**a**) Schematic representation of hippocampal slices incubation protocol. (**b**) Representative traces (1) prior and (2) after LTP induction, composed of the stimulus artifact followed by the presynaptic volley and the fEPSP. (C) Changes in fEPSP slope upon LTP induced by theta-burst stimulation from hippocampal rat slices (CTR: control; aSyn olig:

after pre-incubation with aSyn oligomers, 500 nM, 90 min; aSyn olig + SCH 58261: after incubation with aSyn oligomers in the presence of the $A_{2A}R$ antagonist, SCH 58261, 50 nM, 110 min). SCH 58261 rescued LTP impairment induced by aSyn oligomers. $P < 0.01$. (**d**) LTP magnitude after theta-burst stimulation (change in fEPSP slope at 50–60 min). (**e**) Effect of NMDAR antagonist APV (50 µM, 30 min) superfusion on basal fEPSP slope. SCH 58261 prevented the effect of aSyn oligomers on NMDAR contribution to basal synaptic transmission. $P < 0.05$. (**f**) Quantification of the effects observed in (e) (change in slope between baseline and the last 10 min of APV application). (**g**) Input/Output (I/O) curves corresponding to fEPSP slope evoked by different stimulation intensities (60 – 300 µA). Slices co-incubated with aSyn olig and SCH 58261 displayed higher Emax values when compared with control slices, similar to what was observed with aSyn olig alone. (**h**) Co-immunoprecipitation of PSD-95 in hippocampal slices. NMDAR2B are enriched in aSyn olig pre-incubated slices while co-incubation with SCH 58261 reestablished NMDAR2B subunit levels. NMDAR1 levels were not changed in any condition. Values were normalized to PSD-95. IgG was used as a negative control (Neg CTR). $P < 0.05$. All values are mean ± SEM of 3-9 independent experiments.

We then assessed the synaptotoxic effects of aSyn oligomers on hippocampal slices from $A_{2A}R$ KO mice. Remarkably, aSyn oligomers (500 nM, 90 min) failed to impair LTP in $A_{2A}R$ KO mice (LTP_{CTR} = 34.3 ± 6.9%; $LTP_{aSyn\ olig}$ = 31.2 ± 4.2%; $n = 3$; $P < 0.05$; Fig. 3D-E), in contrast to the significant effect in WT mice (LTP_{CTR} = 64.43 ± 14.2%, $n = 4$; $LTP_{aSyn\ olig}$ = 10.9 ± 12.9%; $n = 3$; $P < 0.05$; **Fig. 2.3a, b**). As observed in rat hippocampal slices, aSyn oligomers also increased basal synaptic excitability, as observed by the shift to the left of the I/O curve, both in WT and $A_{2A}R$ KO mice (**Fig. 2.3c, f**).

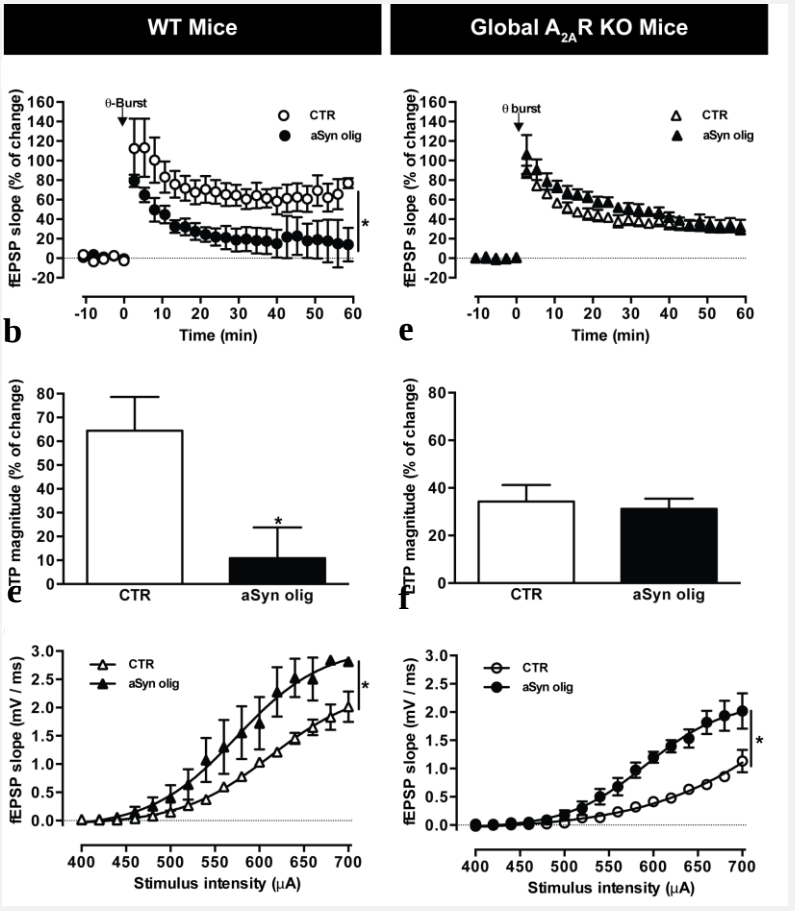


Figure 2.3. A_{2A}R deletion fully prevents LTP impairment induced by extracellular aSyn oligomers. (**a**) Changes in fEPSP slope induced by theta-burst stimulation recorded from WT mice hippocampal slices in control conditions (CTR) or in the presence of aSyn oligomers (aSyn olig, 500 mM, 90 min). (**b**) Changes in fEPSP slope induced by theta-burst stimulation recorded from global A_{2A}R KO mouse hippocampal slices in control conditions or in the presence of aSyn oligomers (aSyn olig). Genetic deletion of A_{2A}R prevented LTP impairment induced by aSyn olig in WT mice slices. (**c**) Plot of the LTP magnitude (change in fEPSP slope at 50–60 min comparing to baseline) from (a). (**d**) Plot of the LTP magnitude from experiments shown in (b). (**e**) I/O curves from WT hippocampal slices, corresponding to fEPSP slope evoked by various stimulation intensities (400

–700 µA) in control conditions and upon pre-incubation with aSyn oligomers. (**f**) I/O curves from $A_{2A}R$ KO hippocampal slices obtained by the same method as in (e). aSyn oligomers have a comparable effect both in WT or in $A_{2A}R$ KO mice. All values are mean ± SEM of 3-4 independent experiments.

$A_{2A}R$ blockade rescues cell death induced by endogenous aSyn

To evaluate the role of $A_{2A}R$ on the toxicity induced by intracellular aSyn, we used an established Dox-inducible (tet-off) neuroblastoma cell model that we verified to express $A_{2A}R$ (**Supplementary Fig. 2.1a**). Maximal aSyn expression was reached at 12 days after Dox removal (**Supplementary Fig. 2.1b**) and, at this time point, we found a significant decrease in cell viability compared to control cells in the presence of Dox (+Dox) (cell viability$_{+Dox\ CTR}$ = 91.6 ± 1.5 %; cell viability$_{–Dox\ CTR}$ = 68.7 ± 1.6%; n = 8-9; P < 0.001; **Fig. 2.4a, b**); the $A_{2A}R$ mRNA or protein levels were not altered (**Supplementary Fig. 2.1c, d**). This toxicity was completely prevented by a 24 h treatment with the selective $A_{2A}R$ antagonists, SCH 58261 or ZM 241385 (cell viability$_{–Dox+SCH\ 58261}$ = 87.1 ± 5.1%; cell viability$_{-Dox+ZM\ 241385}$ = 84.8 ± 5.5%; n = 4-7; P < 0.01 *vs.* +Dox CTR; **Fig. 2.4a, b**). Furthermore, activation of $A_{2A}R$ with the selective $A_{2A}R$ agonist CGS 21680 (30 nM) in control cells in the presence of Dox, resulted in cytotoxicity, mimicking aSyn overexpression (-Dox CTR) (**Fig. 2.4c**). Importantly, these effects were not due to changes in aSyn levels induced by the different treatments (**Supplementary Fig. 2.1e**).

Next, we investigated if NMDAR were involved in this effect. When NMDAR were blocked by APV alone, the toxicity induced by overexpression of aSyn (-Dox; **Fig. 2.4a, c**) was completely rescued (cell viability$_{-Dox+APV}$ = 92.5 ± 1.7%; n = 4; P < 0.001 *vs.* -Dox CTR). This effect is the same of that observed upon $A_{2A}R$ blockade. Consistently, blockade

of NMDAR also prevented toxicity induced by direct A_{2A}R activation with CGS 21680 (**Fig. 2.4a, c**).

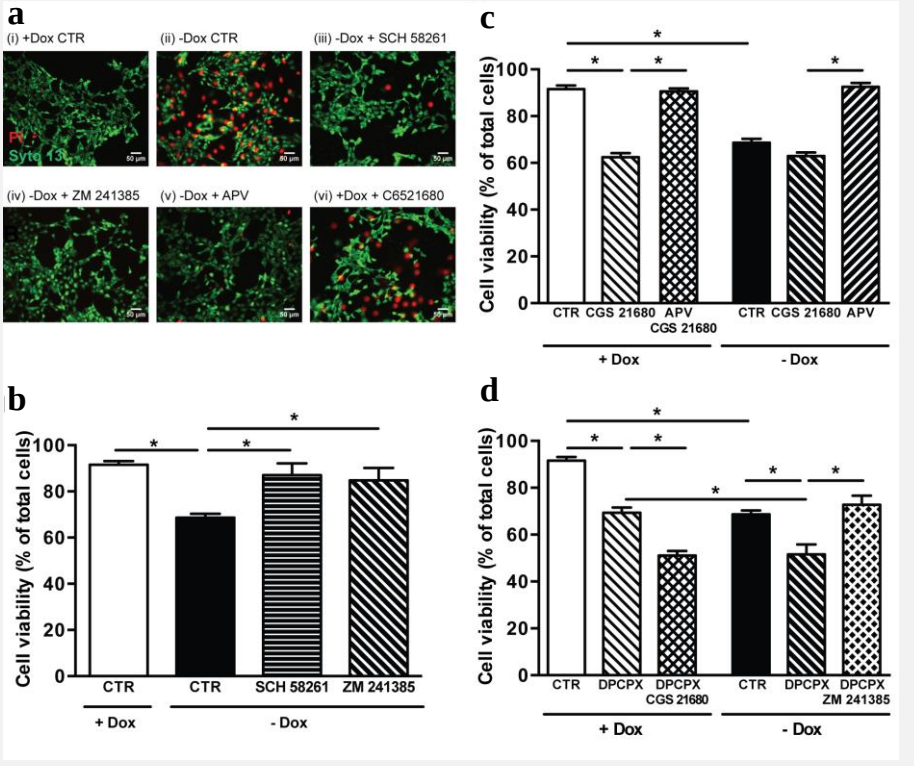


Figure 2.4. A_{2A}R blockade rescues SH-SY5Y cell toxicity induced by increased levels of endogenous aSyn. (**a**) Representative images of 12 days *in vitro* (DIV) tet-off SH-SY5Y cells labeled with PI and Syto-13 (i) in the presence (ii) or absence of doxycycline (Dox; 2 µg/mL) and (iii-vi) under different treatments. Scale bar: 50 µm. (**b**) Cell viability with different cell treatments. The overexpression of aSyn, induced by the absence of Dox (-Dox), lead to a decrease in the number of viable cells, which was prevented by the selective A_{2A}R antagonists, SCH 58261 (50 nM) or ZM 241385 (50 nM). (**c**) Impact of the A_{2A}R agonist, CGS 21680 (30 nM) on cell viability. The treatment of control cells (CTR +Dox) with the A_{2A}R agonist, lead to a similar decrease in cell death as observed in aSyn overexpressing cells (CTR -Dox); this increase in cell death was prevented in both conditions upon

treatment with the NMDAR antagonist, APV (50 μM). (**d**) Impact of the A_1R selective antagonist, DPCPX (50 nM) on cell viability. The treatment with the DPCPX induced a similar decrease in cell viability either in CTR +Dox or aSyn overexpressing cells (CTR -Dox). Cell viability is presented as the ratio between the number of living cells and the number of total cells counted. $P < 0.01$. All values are mean ± SEM.

To rule out any possible contribution of the more abundant adenosine receptor, A_1R, to the aSyn-induced effects, we selectively blocked this receptor (DPCPX; 50 nM).

DPCPX alone (50 nM) reduced cell viability by 20%, as expected (Valadas et al., 2012). A_1R are not involved in aSyn induced toxicity since this reduction was similar in either Dox+ or Dox- cells (**Fig. 2.4d**). Moreover, the effect of $A_{2A}R$ activation by CGS 21680 is independent of A_1R, being maintained even under A_1R blockade (+Dox plus DPCPX+CGS). Finally, the increase in cell viability achieved by $A_{2A}R$ blockade (ZM 241385) in Dox- cells is still present even under A_1R blockade (**Fig. 2.4d**).

$A_{2A}R$ blockade does not affect aSyn oligomerization

We next evaluated whether $A_{2A}R$ affected the initial events of aSyn aggregation, by monitoring the ability of aSyn to oligomerize, using a stable cell model of aSyn dimerization/oligomerization based on BiFC (Outeiro et al., 2008) (**Fig. 2.5a**). We found that aSyn formed oligomers in H4 control cells (CTR; **Fig. 2.5c**) and no significant differences in the oligomerization pattern was detected upon $A_{2A}R$ modulation, as assessed by Venus fluorophore reconstitution (**Fig. 2.5b, c**). This lack of effect does not stem from a reduced dynamic range, as we have previously validated the reversibility of the system in response to multiple conditions (Outeiro et al., 2008; Zondler et al., 2014).

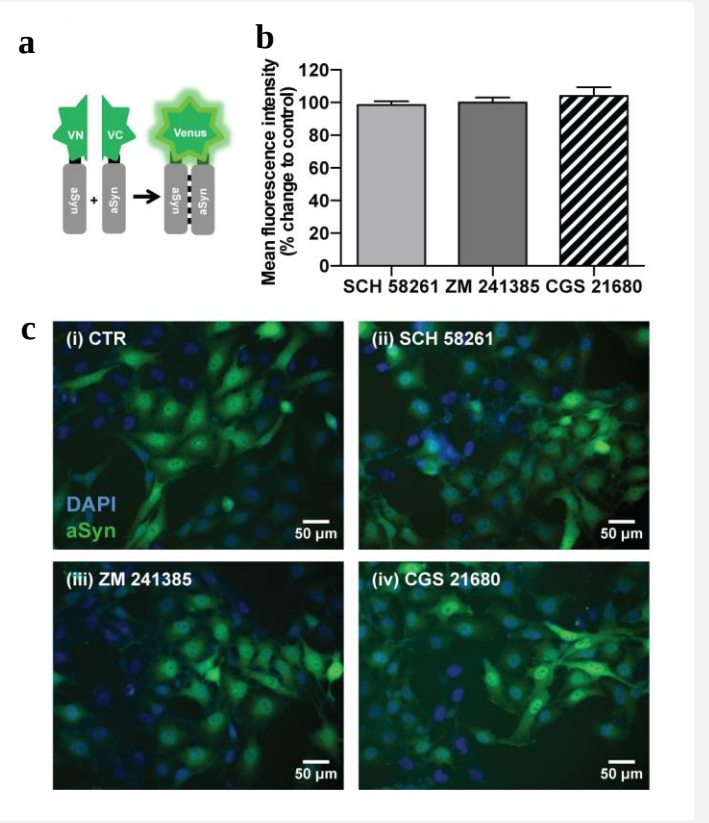


Figure 2.5. $A_{2A}R$ modulators do not change aSyn dimerization in living cells using Bimolecular Fluorescence Complementation (BiFC) assay. (**a**) Schematic representation of the BiFC assay. aSyn BiFC constructs in an anti-parallel orientation. (**b**) Quantification of the mean fluorescence intensity of cells showing no significant difference in cells treated with the $A_{2A}R$ antagonists ZM 241385 (50 nM) or SCH 58261 (50 nM) nor with the $A_{2A}R$ agonist CGS 21680 (30 nM). Results were normalized to control condition (CTR). All values are mean ± SEM of 5 independent experiments. (**c**) Representative images of H4 cells with different treatments. Scale bar: 50 µm.

$A_{2A}R$ blockade decreases aSyn aggregation

Finally, we asked whether $A_{2A}R$ affected latter steps in the process of aSyn aggregation. For this, we used an established model of aSyn inclusion formation in human neuroglioma H4 cells (Lázaro et al., 2014; McLean et

al., 2001) that also express $A_{2A}R$ (**Fig. 2.6c, d**). We found that a 24-h treatment with the $A_{2A}R$ antagonist ZM 241385 (50 nM) significantly reduced the percentage of cells displaying aSyn inclusions (aSyn $inclusions_{CTR}$ = 100.0 ± 2.9%; aSyn $inclusions_{ZM\ 241385}$ = 81.8 ± 5.9%, *n* = 3-4; *P* < 0.05; **Fig. 2.6a, b**). In contrast, activation of $A_{2A}R$ with CGS 21680 (30 nM) increased the percentage of cells containing aSyn inclusions (aSyn $inclusions_{CGS\ 21680}$ = 117.3 ± 1.5%, *n* = 3; *P* < 0.05 *vs.* aSyn $inclusions_{CTR}$; **Fig. 2.6a, b**).

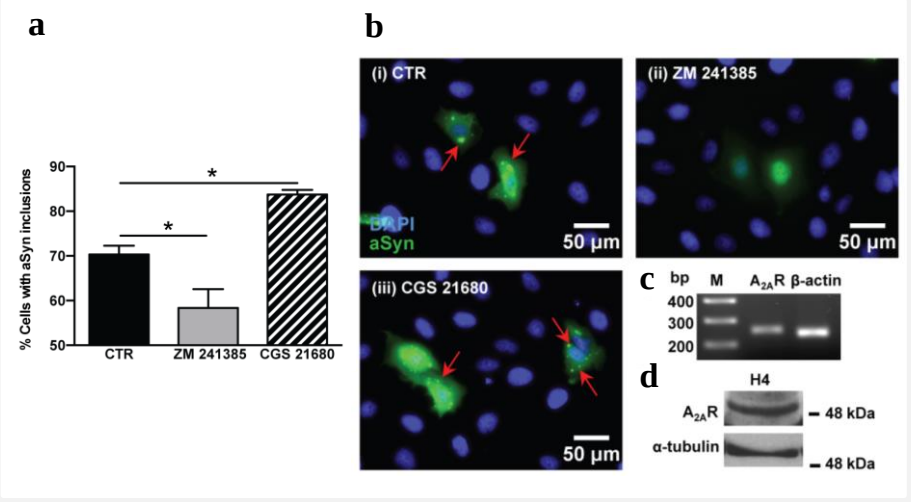


Figure 6. $A_{2A}R$ blockade decreases aSyn aggregation in a SynT-Synphilin-1 neuroglioma cell model. (**a**) Representative images of H4 cells in (i) control conditions (CTR), (ii) incubated for 24 h with ZM 241385 (50 nM) or (iii) CGS 21680 (30 nM). Scale bar: 50 µm. (**b**) ZM 241385 significantly reduced the % of cells containing aSyn inclusions whereas CGS 21680 increased the % of cells with inclusions, compared with control cells. Results are expressed as the percentage of the total number of transfected cells. *P* < 0.05. All values are mean ± SEM of 3 independent experiments. (**c**) qPCR products of $A_{2A}R$ (245 bp) in H4 cells, β-actin (233 bp) was used as housekeeping control and RT-minus control yielded no appreciable bands in the expected band size for the primers used. (**d**) Western blot showing $A_{2A}R$ expression in H4 cells.

Discussion

We have gathered evidence indicating that $A_{2A}R$ play an important role in modulating the deleterious effects of aSyn. Here, we show, for the first time, that selective $A_{2A}R$ antagonists are able to rescue both exogenous and intracellular aSyn-mediated cyto- and neurotoxicity. Furthermore, pharmacological and genetic inactivation of $A_{2A}R$ fully prevents the aSyn-mediated toxic effects on synaptic function. This neuroprotective effect afforded by $A_{2A}R$ inhibition is due to the reestablishment of glutamate NMDAR signaling. Finally, we found that $A_{2A}R$ antagonists are also able to decrease the number of aSyn aggregates, which might explain their protective effects on aSyn-associated synaptic dysfunction and neuronal death.

aSyn aggregation and progressive neuronal cell death are the neuropathological hallmarks of several neurodegenerative disorders known as synucleinopathies (Marques and Outeiro, 2012). However, the precise molecular mechanisms underlying the process of aSyn aggregation and the exact nature of the toxic species produced during aggregation remain unclear. aSyn is a soluble protein expressed ubiquitously in the CNS, including the cerebral cortex, hippocampus, amygdala, and olfactory bulb (Abeliovich et al., 2000; Maroteaux and Scheller, 1991). Under pathological conditions, this protein exhibits a propensity to misfold and aggregate, first into small oligomeric species that are rich in β-sheet structure, and then into higher molecular weight insoluble fibrils (Lashuel et al., 2012; Spillantini et al., 1998). As reported for amyloid-beta (Aβ) plaques in AD, soluble oligomeric species are thought to constitute the most neurotoxic species (Emadi et al., 2009; Glabe and Kayed, 2006; Irvine et al., 2008; Kayed et al., 2003; Martin et al., 2012; Outeiro et al., 2008). Consistently, we observed that mature extracellular aSyn oligomers have the ability to induce synaptic impairment through an NMDAR-

dependent mechanism (Diógenes et al., 2012). We now report that exposure to aSyn oligomers causes damage in primary cortical cultures, ultimately leading to neuronal death. In contrast, aSyn monomers have no effect.

An imbalance in adenosine levels in the brain together with an abnormal function and increased $A_{2A}R$ levels, which facilitates excitotoxicity and consequent neuronal death, has been reported in multiple conditions such as ischemia, stress, epilepsy, AD and PD (Batalha et al., 2013; Cunha et al., 2006; Latini and Pedata, 2001; Lopes et al., 1999a; Rebola et al., 2005b; Varani et al., 2010; Villar-Menéndez et al., 2014). Based on this idea, $A_{2A}R$ antagonists started emerging as promising candidates in modulating the demise of different psychiatric and neurological disorders, including PD. In fact, it was demonstrated that consumption of caffeine, a non-specific $A_{2A}R$ antagonist, reduces the risk of developing PD (Ascherio et al., 2001). Indeed, polymorphisms in the human $A_{2A}R$ gene (ADORA2A) are linked to a reduced risk of Parkinson's disease (Popat et al., 2011). The specific blockade of these receptors was shown to be protective in several Parkinson's disease models (Aguiar et al., 2006; Chen et al., 2001a; Ikeda et al., 2002; Kachroo et al., 2010; Xu et al., 2010); including in aSyn-mediated neurotoxicity (Kachroo and Schwarzschild, 2012). The crossing of $A_{2A}R$ KO with *hm*^{2}-aSyn mice resulted in reduced neuronal loss, suggesting the potential involvement of $A_{2A}R$ on aSyn-associated toxicity. However, in this report, the impact of $A_{2A}R$ on aSyn oligomerization or aggregation was not determined (Kachroo and Schwarzschild, 2012).

In order to clarify the molecular basis of $A_{2A}R$-mediated protection against aSyn toxicity, we now evaluated the $A_{2A}R$ effect in multiple models of aSyn aggregation and toxicity. Our data demonstrate that the selective blockade or deletion of $A_{2A}R$ prevents both synaptic plasticity impairment and neuronal death induced by extracellular aSyn oligomers.

The ability to respond to theta-burst stimulation, when exposed to aSyn mature oligomers, is restored either in hippocampal slices from $A_{2A}R$ KO mice or, WT slices in the presence of a specific $A_{2A}R$ antagonist. As we have previously reported, aSyn oligomers promote an increase in basal synaptic transmission both by the activation of NMDAR and by the insertion of Ca^{2+}- permeable AMPA receptors in the postsynaptic membrane (Diógenes et al., 2012), which leads to synapse saturation and consequent LTP impairment. Since Ca^{2+}- permeable AMPA receptors are crucial for LTP maintenance (Plant et al., 2006), the complete rescue of LTP impairment by $A_{2A}R$ antagonist suggests a reestablishment of this AMPA impaired trafficking. In fact, it has been described that $A_{2A}R$ have the ability to modulate the membrane levels of Ca^{2+}-permeable AMPA receptors (Dias et al., 2012), which can explain the observed effects. Furthermore, we show that the basal overactivation of NMDAR caused by aSyn oligomers is also prevented by $A_{2A}R$ blockade since NMDAR basal contribution is no longer observed. However, while the glutamatergic transmission is restored, the effects of aSyn on basal synaptic transmission were not rescued by $A_{2A}R$ blockade or in $A_{2A}R$ KO mice, as reflected in the unmodified steeper I/O curve. $A_{2A}R$ do not only affect glutamatergic transmission but can also directly enhance inhibitory GABAergic transmission, leading to disinhibition of pyramidal cells (Rombo et al., 2014). A possible explanation for the lack of effect of $A_{2A}R$ blockade on the I/O curve might be due to a resulting overall excitation, caused by a decrease in the inhibitory GABAergic tonus.

In addition to these effects in early synaptic dysfunction, $A_{2A}R$ antagonists were also effective in preventing subsequent neuronal death in neuronal cultures exposed to aSyn oligomers. Our observations are consistent with previous reports showing that pharmacologic or genetic modulation of $A_{2A}R$ can prevent neurotoxicity and the extent of neuronal damage in neurons affected by ischemia, hypoxia, stress or Aβ exposure

(Canas et al., 2009; Cunha, 2005; Valadas et al., 2012). To further detail the mechanism of $A_{2A}R$ action on aSyn-induced toxicity, we used an established SH-SY5Y cell model of endogenous aSyn accumulation (Vekrellis et al., 2009). Cells overexpressing aSyn for 12 DIV show increased cell death that is rescued by selective $A_{2A}R$ antagonists. Furthermore, if we activate $A_{2A}R$ in control cells (+Dox), we mimic the cytotoxic effects induced by aSyn accumulation (-Dox), supporting the idea of the involvement of $A_{2A}R$ overactivation on aSyn-induced cell death. Together these data raise the hypothesis that aSyn accumulation is leading to a toxic overactivation of $A_{2A}R$. This can result either from, overexpression of $A_{2A}R$ in these conditions or, alternatively, to an increase of the endogenous ligand adenosine. The fact that overexpression of aSyn does not alter $A_{2A}R$ levels favors the latter hypothesis. Under physiological conditions, adenosine is tonically activating the abundant A_1R and producing synaptic inhibition (Dittman and Regehr, 1996; Dunwiddie and Diao, 1994; Takahashi et al., 1995). However, in this situation, A_1R do not seem to contribute to aSyn-induced toxicity, since when we blocked the receptors, we could not see any differences in cell viability upon aSyn accumulation. This is in accordance with different sources of adenosine activating A_1R and $A_{2A}R$ (Cunha, 2008).

Together, our results suggest that aSyn-induced cell death is associated with an increase in $A_{2A}R$ activation that mediates NMDAR overactivation which is a prominent synaptic event leading to excitotoxicity (Besancon et al., 2008). In fact, $A_{2A}R$ are known to increase NMDAR function in the hippocampus (Rebola et al., 2008), namely promoting Ca^{2+} entry through NMDAR, by (PKA)-dependent regulation (Higley and Sabatini, 2010). Interestingly, the same mechanisms as Ca^{2+} entry dysfunction (Martin et al., 2012) and NMDAR activation (Diógenes et al., 2012) are involved in aSyn-associated neurotoxicity. This suggests that $A_{2A}R$ blockade is probably counteracting these aSyn-associated

effects, which then translates into the prevention of synaptic dysfunction and cell death. While non-neuronal $A_{2A}R$ have been implicated in neurotoxin-based PD mechanisms (such as MPTP or 6-OHDA) (Yu et al., 2008), forebrain neuronal $A_{2A}R$ are critical for the control of cortico-striatal synaptic activity. Our data reinforce the notion that counteracting neuronal $A_{2A}R$ activation has benefits, specifically against aSyn-induced synaptic deficits.

Furthermore, dysfunction in Ca^{2+} has also been shown to increase aSyn propensity to form aggregates (Rcom-H'cheo-Gauthier et al., 2014) which are known to be associated with PD-related neurotoxicity (Conway et al., 2000; Karpinar et al., 2009). Based on these findings, we hypothesized that the observed protective effects of $A_{2A}R$ blockade could be due to the modulation of aSyn aggregation process, as also observed to occur for the formation of mutated ataxin-3 aggregates (Gonçalves et al., 2013). To this end, we used two cell-based models mimicking different steps of the aSyn aggregation process namely dimerization/ oligomerization and inclusions formation (McLean et al., 2001, 2002; Outeiro et al., 2008). We did not detect significant differences in the oligomerization pattern using the BiFC assay, upon treatment with $A_{2A}R$ modulators. Since this assay does not distinguish between dimers, trimers and higher molecular weight aSyn oligomers (Outeiro et al., 2008), we cannot discard the hypothesis that $A_{2A}R$ modulators might interfere with the later stages of aggregation. Indeed, $A_{2A}R$ blockade decreases the number of aSyn inclusions in a cell model of aSyn inclusion formation, while their activation enhances inclusion formation. It has been reported that the activation of NMDAR can downregulate the ubiquitin proteasome system (Caldeira et al., 2013), which may consequently lead to the accumulation of proteins that are prone to aggregation, like aSyn. There are reports suggesting that $A_{2A}R$ can directly bind and modulate the activity of ubiquitin proteasome system (Chiang et al., 2009; Milojevic et

al., 2006). The observed changes are not associated with $A_{2A}R$ affecting directly the levels of aSyn, in accordance with previous observations from Kachroo and Schwarzschild et al (2012). In that study, the authors did not assess changes in the aggregation pattern. Here, we now report for the first time, that the $A_{2A}R$ blockade reduces the percentage of cells containing inclusions, using a very sensitive model that allows more accurate quantifications. Our results suggest that $A_{2A}R$ modulation does not interfere with the initial events leading to the formation of oligomeric species but, instead, may interfere with the latter stages of the aSyn aggregation process.

Currently, there are multiple specific $A_{2A}R$ antagonists, including caffeine, progressing through phase II and III clinical trials for the symptomatic treatment of PD (Barkhoudarian and Schwarzschild, 2011). Thus, this class of agents is well positioned for clinical testing of their neuroprotective potential. The present findings strengthen the rationale for disease modification trials of $A_{2A}R$ antagonism and complement epidemiological data on caffeine links to a reduced risk of PD, and substantially broaden the potential use of $A_{2A}R$ as therapeutic targets in synucleinopathies.

Overall, our results highlight the interplay between toxic and protective influences of $A_{2A}R$ on aSyn aggregation and associated synaptic toxicity and neurodegeneration, raising the possibility that adenosine $A_{2A}R$ antagonists produce their well-documented neuroprotective effects in PD models by preventing aSyn-inclusion formation and consequent associated toxicity. Furthermore, we now show that this rescue of aSyn-associated toxicity is being mediated via NMDAR, which are known to be involved in proteasome clearance system. Moreover, we also demonstrate that both intracellular overexpression of aSyn and extracellular addition of oligomeric aSyn increases cell death, suggesting that aSyn may induce similar toxic effects irrespective of being generated intra- or extracellulary

and more importantly $A_{2A}R$ antagonists are able to completely rescue these aSyn-associated toxic events.

Materials and Methods

Animals

Animal procedures were performed in accordance with the guidelines of the European Community guidelines (Directive 2010/63/EU), Portuguese law on animal care (1005/92) and approved by the *Instituto de Medicina Molecular* Internal Committee and the Portuguese Animal Ethics Committee (*Direcção Geral de Veterinária*). Environmental conditions were kept constant: food and water *ad libitum*, 21 ± 0.5°C, 60 ± 10% relative humidity, 12-h light/dark cycles. All animals were killed by decapitation after anesthesia under halothane atmosphere. Male Wistar rats (8–12 weeks old) purchased from Harlan Interfauna Iberica, SL. Global $A_{2A}R$ KO mice with a C57Bl/6-background were generated by a standard replacement-type vector constructed to inactivate the $A_{2A}R$ (Chen et al., 1999). Congenic global $A_{2A}R$ KO mice were made by backcrossing KO on mixed (129-Steel x C57BL/6) genetic background to C57BL/6 mice for 13–15 generations. Heterozygous cross-breeding was used to generate WT and global KO mice. Male KO and WT mice with matched age (8-12 weeks old, male) were used for electrophysiological experiments.

Purification and Oligomerization of Recombinant aSyn

aSyn was prepared as previously (Diógenes et al., 2012; Vicente Miranda et al., 2013). Monomeric aSyn was readily used or stored at -80°C until further use. Oligomerization was induced by the continuous shaking of monomeric aSyn (140 µM) for 6 days at 37°C in a thermomixer (Eppendorf) at 900 rpm. Samples were ultracentrifuged to remove fibrillary aSyn. The supernatant containing monomeric and oligomeric aSyn was centrifuged in Amicon filter unit with Ultracel membrane NMWL of 30 kDa (Millipore). The retained fraction, containing aSyn

oligomers (> 30 kDa) was readily used or stored at -80°C until further use. The concentration of aSyn was determined using its molar extinction coefficient at 280 nm (i.e. ε_{280}=5960 L/mol/cm).

SDS-PAGE

The composition of different aSyn species, monomers, and oligomers, was evaluated by SDS-PAGE. Five micrograms of each aSyn sample was separated by SDS-PAGE using a Tetra Cell (Bio-Rad) on a precast 4–15% polyacrylamide gel (Bio-Rad) using standard procedures. Proteins were transferred to a nitrocellulose membrane (Bio-Rad) using the Mini Tans-Blot system (Bio-Rad).

Prestained standard proteins were also loaded on the gel. Membrane was blocked for 1 h at room temperature (RT) with blocking solution (5% bovine serum albumin in 50 mM Tris, 150 mM NaCl, 0.1% and Tween 20, pH 7.5). The membrane was incubated overnight at 4°C with the mouse anti-aSyn primary antibody (1:1000; BD transduction lab) diluted in blocking solution. Membrane was washed and incubated for 1 h at RT with anti-mouse-horseradish peroxide (HRP)-conjugated secondary antibody (1:10000, Invitrogen) diluted in blocking solution. Detection procedures were performed according to ECL system (Millipore) using a chemidoc system (Bio-Rad).

Rat Primary Neuronal Cultures

Hippocampal neurons were cultured from 18 days Sprague Dawley rat (Harlan, Barcelona, Spain) embryos as previously described (Valadas et al., 2012). Briefly, embryos were collected in Hank's Balanced Salt Solution (1 mM Ca^{2+} and 1 mM Mg^{2+}) and rapidly decapitated. Meninges and white matter were removed and whole cortices (hippocampi and attached cortex) were incubated for 15 min in Hank's Balanced Salt

Solution and 0.025% trypsin. Cells were centrifuged three times and washed with Hank's Balanced Salt Solution (10% fetal bovine serum) and finally resuspended in Neurobasal medium. Cells were plated on poly-D-lysine-coated coverslips in 24-well plates at a density of 8 x 10^4 cells/well. Neurons were grown for 10 days at 37°C in a 5% CO_2 humidified atmosphere in Neurobasal medium with 2% B-27 supplement, 25 μM glutamate, 0.5 mM glutamine, and 2 U/mL penicillin/streptomycin, in the absence of any positive selection for neurons. Cells with 9 or 10 DIV were treated with extracellular aSyn species (500 nM) for 24 h or 90 min, respectively.

Electrophysiological fEPSPs Recordings

The experiments were performed in acute transverse hippocampal slices from male Wistar rats (8–12 weeks old) and in $A_{2A}R$ KO and WT mice. After decapitation, the brain was rapidly removed, and the hippocampi were dissected free in ice-cold artificial CSF or Krebs solution composed of (mM): NaCl 124; KCl 3; NaH_2PO_4 1.25; $NaHCO_3$ 26; $MgSO_4$ 1; $CaCl_2$ 2; and D-glucose 10, previously gassed with 95% O_2 and 5% CO_2, pH 7.4. Slices (400 μm thick) were obtained with a McEwan tissue chopper and were incubated with or without extracellular aSyn oligomers (500 nM) for 90 min at RT in gassed artificial CSF. Incubation with SCH 58261 (50 nM) started 20 min prior to aSyn oligomers incubation and was kept throughout the 90 min of aSyn incubation (**Fig. 2.2a**). Following this incubation period, slices were superfused with artificial CSF (3 mL/min) at 32ºC and fEPSPs were recorded as previously (Diógenes et al., 2012) in the *stratum radiatum* of the CA1 area. We first carried out input–output (I/O) curves and then LTP was induced by a theta-burst protocol (10 trains with four pulses each at 100 Hz, separated by 200 ms).

SH-SY5Y Cells Inducibly Overexpressing Wild Type aSyn

Stable SH-SY5Y cell lines inducibly expressing human WT aSyn (a kind gift from Prof. Kostas Vekrellis, Athens, Greece) were generated as previously described (Vekrellis et al., 2009). Cells were cultured in RPMI 1640® medium (Life Technologies) supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 mg/mL). SH-SY5Y cells were maintained in 250 μg/mL G418 and 50 μg/mL hygromycin B. aSyn expression was switched off by the addition of doxycycline (Dox) (2 μg/mL). Stock cultures were kept in the presence of Dox. Overexpressing aSyn cells were maintained for 12 DIV in the absence of Dox. For propidium iodide (PI) and Syto-13 uptake assay cells were plated onto 12-well plates (3.8 cm^2) at a density of 6 x 10^4 cells/well, 24 h before drug exposure. For Western blot analysis, the cells were seeded into 6 well plates at a density of 15 x 10^4 cells/well.

H4 Cells Stably Expressing VN-Syn/Syn-VC

For the aSyn dimerization model, human H4 neuroglioma cells stably expressing two aSyn BiFC constructs were used (Outeiro et al., 2008). This assay is based on the reconstitution of functional fluorescent proteins promoted by the interaction between, at least, two aSyn molecules, that enables the direct visualization of aSyn dimeric/oligomeric species formation. The two BiFC constructs used were generated by fusing half of the fluorescent Venus protein with aSyn in the N-terminal and the other half fused with aSyn in the C-terminal.

Cells were maintained in OPTI-MEM® (Life Technologies) supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 mg/mL) in a humidified atmosphere of 5% of CO_2 in air at 37°C. Cells were plated onto 12-well plates and 24 h before treatment. 24 h after drug treatment, cells were washed with phosphate

buffer saline (PBS: NaCl 137 mM, KCl 2.7 mM, KH_2PO_4 1.8 mM and Na_2HPO_4 10 mM, pH 7.4) and fixed with 4% paraformaldehyde (PFA) for 10 min at RT, followed by a 10 min incubation with Hoescht 33258 dye (1 mg/mL, Life Technologies-Invitrogen, Carlsbad, CA, USA) at RT. Cells were then washed and maintained in PBS and imaged on an Olympus IX81-ZDC microscope system (Olympus Germany, Hamburg, Germany) using the 20x objective and maintaining the same exposure time for Venus and Hoechst channels for each condition. Quantification of the number of cells and average Venus fluorescence intensity was performed using an in-house developed macro for ImageJ (http://imagej.nih.gov/ij/). Briefly, single cell nuclei were identified using the Hoechst channel by thresholding and particle analysis and the corresponding regions of interest (ROIs) were then used to measure the average intensity in the Venus channel. The number of cells with aSyn dimers was determined by counting the ROIs where the average fluorescent intensity was higher than a given threshold. Values of each condition were then averaged, and statistical analysis was performed.

Human Neuroglioma H4 Cells

Human neuroglioma H4 cells were maintained in OPTI-MEM® (Life Technologies) supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 mg/mL) in a humidified atmosphere of 5% of CO_2 in air at 37 °C. Cells were plated in 12-well plates 24 h prior to transfection. Cells were transfected with equimolar amounts of the plasmids encoding the human WT aSyn with a C-terminal tag corresponding to a truncated fragment of EGFP (referred to as SynT) and synphilin-1 as previously (Lázaro et al., 2014). 24 h after transfection, cells were incubated for 24 h with different $A_{2A}R$ modulators and, after this period, cells were subjected to immunocytochemistry for studying aSyn

inclusions. Transfected cells were identified and classified into two groups: cells without inclusions and cells with one or more inclusions. Results were expressed as the percentage of the total number of transfected cells.

Immunocytochemistry

48 h after transfection, H4 cells were washed with PBS and fixed with 4% PFA for 10 min at RT, followed by a permeabilization step with 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 20 min at RT. After blocking in 1.5% normal goat serum (PAA, Cölbe, Germany)/DPBS for 1 h, cells were incubated with mouse anti-aSyn primary antibody (1:1000, BD Transduction Laboratory, New Jersey, USA) primary antibody overnight at 4ºC. After a 30 min washing with PBS, cells were incubated with the secondary antibody Alexa Fluor 488 donkey anti-mouse IgG (Life Technologies- Invitrogen, Carlsbad, CA, USA) for 2 h at RT. Finally, cells were stained with Hoechst 33258 (1 mg/mL, Life Technologies-Invitrogen, Carlsbad, CA, USA) (1:5000 in DPBS) for 10 min, and maintained in PBS for epifluorescence microscopy.

PI and Syto-13 Uptake Assay

This protocol was used either in primary cortical cultures or SH-SY5Y cells and performed as previously described (Valadas et al., 2012). Briefly, cells were washed with Krebs-HEPES (NaCl 117 mM, KCl 3 mM, glucose 10 mM, $NaHCO_3$ 26 mM, Na_2HPO_4 1.25 mM, HEPES 10 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM), incubated with Syto-13 (4 μM) and PI (5 μg/mL) for 3 min at RT and directly observed using an Axiovert 200 fluorescence microscope. An average of 1400 cells was counted per condition in each experiment. Syto-13 labels with green fluorescence (emits preferentially at 509 nm when excited at 488 nm) both RNA and DNA in living cells. PI

labels with red fluorescence (absorbing preferentially at 535 nm and emitting at 617 nm) cells that lost plasma membrane integrity. Cell viability was presented as the ratio between the number of living cells and the total number of cells.

Real-Time qPCR

Total RNA from H4 and SH-SY5Y cells was extracted using the RNAspin Mini RNA isolation kit (GE Healthcare, Buckinghamshire, UK). Briefly, cell cultures were washed with PBS, scraped, collected in lysis buffer and processed according to the manufacturer's instructions. RNA was quantified with the NanoDrop 2000 (Thermo scientific, Wilmington, DE, USA). Total RNA (2 μg) was reverse-transcribed using random primers and SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Life technologies, Carlsbad, CA, USA) and negative controls were made without reverse transcriptase. qPCR was carried out with Power SYBR Green PCR Master mix (Applied Biosystems, Warrington, UK), using 8 ng/μL of total cDNA and 0.2 μM of each primer, performed in a Rotor-Gene 6000 Real Time Rotary Analyzer (Corbett Research, Cambridge, UK). The thermal cycler conditions were 10 min at 95°C, 40 cycles of a two-step PCR, 95°C for 15 s followed by 60°C for 25 s with a final thermal ramp from 72 to 95ºC. The primers used in qPCR include: forward 5′-AACCTGCAGAACGTCAC-3′ and reverse 5′-GTCACCAAGCCATTGTACCG-3′ for human $A_{2A}R$ (Invitrogen, HPLC purified, product size 245 bp) and forward 5′-GGACTTCGAGCAAGAGATGG-3′ and reverse 5′-AGCACTGTGTTGGCGTACAG-3′ for human β-actin (Invitrogen, HPLC purified, product size 233 bp). The qPCR products were analyzed by electrophoresis on a 2% agarose gel containing Greensafe Premium Nucleic Acid Gel Stain (Nzytech, Portugal).

Co-immunoprecipitation (Co-IP)

Briefly, WT rat hippocampal slices were homogenized in IP buffer (NP40 1%, SDS 0.1%, Tris-HCl 50 mM, NaCl 150 mM, sodium deoxycholate 0,5%, EDTA 1 mM, protease inhibitors - Complete, EDTA-free Protease Inhibitor cocktail tablets; Roche, Manheim, Germany). Protein extracts were incubated with protein G PLUS-Agarose (Santa Cruz Biotechnology, Heidelberg, Germany) for 1 h at 4°C to eliminate non-specific binding. After incubation, the pre-cleared supernatants containing 1 mg protein were incubated with anti-PSD-95 antibody (1:50; Cell Signaling Technology) or IgG (for negative control; Santa Cruz Biotechnology, Heidelberg, Germany) overnight at 4°C under rotation. The day after, lysates were incubated with protein G PLUS-Agarose for 3 h with rotation at 4ºC. Beads were washed three times with IP buffer and resuspended in 1.5x sample buffer pH (Tris 359 mM pH 6.8, glycerol 30%, sodium dodecyl sulfate 10%, dithiothreitol 600 mM, and bromophenol blue 0.012%). Bound proteins eluted from the immune complexes were denatured by heating to 95°C for 5 min and used for Western blot analysis. Western blots were performed with anti-NMDAR2B (1:1000; Cell Signaling technology), anti-NMDAR1 (1:500; BD PharmingenTM) and anti-PSD-95 (1:1000; Cell Signaling Technology) (see Western Blotting).

Western Blotting

SH-SY5Y cells were washed with cold PBS and then mechanically scrapped in radioimmunoprecipitation assay buffer pH 8.0 (RIPA buffer: NaCl 150 mM, Tris-base 50 mM, EDTA 1 mM, Nonidet P40 1%, sodium dodecyl sulfate 0.1%, proteases inhibitors - Complete, EDTA-free Protease Inhibitor cocktail tablets; Roche, Manheim, Germany). Cells were centrifuged at 16000 x *g* during 10 min at 4°C and the pellet, including cell debris, was discarded and the supernatant used for Western

blot. After protein quantification using BioRad *DC* Protein Assay kit, lysates were denatured with 5x sample buffer pH 6.8 (Tris 359 mM pH 6.8, glycerol 30%, sodium dodecyl sulfate 10%, dithiothreitol 600 mM, and bromophenol blue 0.012%) and heated at 95°C for 5 min and further processed as before (Valadas et al., 2012). Samples and the pre-stained molecular weight marker (BIO-RAD) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 15% gel) under reducing conditions and electro-transferred to polyvinylidene difluoride membranes (0.45 μm, Immobilon) using standard procedures. Thereafter, nonspecific binding was blocked with 3% bovine serum albumin (fatty acid-free) in Tris-buffered saline (pH 7.6) containing 0.1% Tween 20 (TBS-T) for 1 h at RT. Membranes were then incubated overnight at 4°C with the corresponding primary antibody, namely mouse anti-aSyn (1:1000; BD transduction lab, Franklin Lakes, NJ, USA), mouse anti-adenosine $A_{2A}R$ (1:1000; Millipore), rabbit anti-α-tubulin (1:5000; Abcam, UK), mouse anti-GAPDH (1:1000; Ambion), rabbit anti-PSD-95 (1:1000; Cell Signaling), rabbit anti-NMDAR2B (1:1000; Cell Signaling), mouse anti-NMDAR1 (1:500; BD Pharmingen^{TM}) diluted in blocking solution. After three washing periods of 10 min with TBS-T, membranes were incubated with horseradish peroxidase (HRP) conjugated anti-mouse or anti-rabbit secondary antibodies (1:10000; Santa Cruz Biotechnology, Heidelberg, Germany) (in 5% non-fat dry milk) for 1 h at RT. After 40 min of washing with TBS-T, chemiluminescent detection was performed with ECL western blotting detection reagent (GE Healthcare) using X-Ray films (Fujifilm, Dusseldorf, Germany). Densitometric quantification was determined using Image-J software and normalized to the corresponding α-tubulin band density.

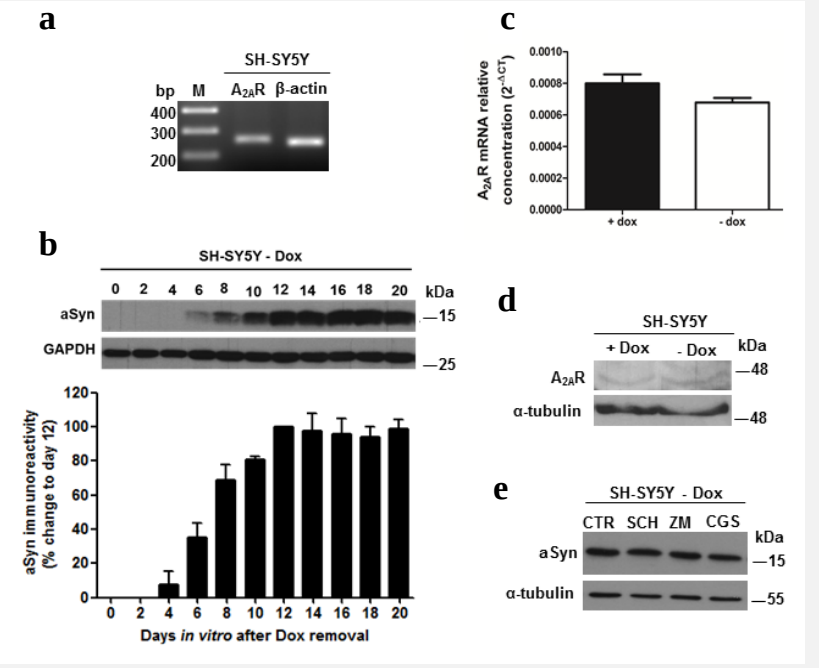
Drugs

5-Amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c] pyrimidine (SCH 58261; Tocris Cookson, UK) and 4-(2-[7-amino)]-2-(2-furyl{1,2,4}-triazolo{2,3-a{1,3,5}triazin-5-yl-aminoethyl)phenol} (ZM 241385; Tocris Cookson, UK) were prepared as 5 mM stock solutions in dimethylsulfoxide (DMSO). 2-[*p*-(2-Carboxyethyl)-phenylethylamino]-5´-*N*-ethylcarboxamidoadenosine (CGS 21680; Tocris Cookson, UK) was prepared as a 12 μM stock solution in DMSO. N^6-Cyclopentyladenosine (CPA; Tocris) was prepared as a 5 mM stock solution in DMSO. 1,3-Dipropyl-8-cyclopentyladenosine (DPCPX; Tocris Cookson, UK) was prepared as a 5 mM stock solution in 99% DMSO and 1% NaOH 1 M. DL-2-Amino-5-phosphonopentanoic acid (APV; Abcam, UK) was prepared as a 100 mM stock solution in NaOH 100 mM. All aliquots were kept frozen at -20°C until use.

Statistics

The values presented are mean ± SEM of *n* independent experiments. To test the significance of the differences between two conditions a Student´s *t* test was used. In statistical tests between three or more conditions, a one-way ANOVA, followed by a Bonferroni's multiple comparison post-hoc test was used. Values of $P < 0.05$ were considered statistically significant.

Supplementary information



Supplementary Figure 1. Characterization of aSyn and $A_{2A}R$ levels in Tet-inducible SH-SY5Y cells. (**a**) qPCR products showing expression of $A_{2A}R$ (245 bp) in SH-SY5Y cells. β-actin (233 bp) was used as a housekeeping control. RT-minus yielded no appreciable bands in the expected band size for the primers used. (**b**) Representative Western blot and averages of three independent experiments showing aSyn levels in the presence (+) or absence (-) of doxycycline (Dox). In the absence of Dox, cells begin expressing aSyn transgene at 6 DIV, this expression increases until 12 DIV, when maximal expression is observed. GAPDH was used as a loading control. (**c**) qPCR showing no change in $A_{2A}R$ mRNA expression levels upon aSyn overexpression. (**d**) Western blot showing no change in $A_{2A}R$ protein levels upon aSyn overexpression. (**e**) Representative Western blot of four independent experiments to evaluate aSyn levels after treatment with SCH 58261 (50 nM), ZM 241385 (50 nM) or CGS 21680 (30 nM). aSyn protein levels remained unchanged in all conditions. α-tubulin was used as a loading control.

Chapter III

Adenosine A_{2A} Receptors Contribute to aSyn-Induced Cognitive Impairment Mediated by PrP^{C} Through mGluR5 and NMDAR2B

Diana G. Ferreira. performed the experimental work, analyzed data and wrote the manuscript, except for some of the IHC experiments (Inês Marques-Morgado) and the production and characterization of aSyn species (Hugo Vicente Miranda).

Abstract

Synucleinopathies, such as Parkinson's disease (PD) and dementia with Lewy bodies (DLB), are neurodegenerative disorders characterized by the accumulation of alpha-synuclein (aSyn) in intracellular inclusions known as Lewy bodies (LB). LBs accumulate throughout the brain as the disease progresses, but the precise significance of these inclusions in disease pathogenesis is still unclear. Currently, prefibrillar, soluble aSyn oligomers, rather than larger inclusions, are considered early and key intermediates in the disease-related synaptic dysfunction that is common to various synucleinopathies. Here, we identified the cellular prion protein (PrP^C) as a key mediator of aSyn-associated synaptic impairment. The aSyn-associated impairment of long-term potentiation (LTP) was blocked in *Prnp* null mice (*Prnp*$^{-/-}$) and rescued upon PrP^C blockade. We show that extracellular aSyn oligomers form a complex with PrP^C at the post-synaptic density, inducing the phosphorylation of intracellular Fyn kinase via metabotropic glutamate receptor 5 (mGluR5). aSyn engagement of PrP^C/Fyn activates NMDA receptor (NMDAR) and alters Ca^{2+} homeostasis. *In vivo* blockade of mGluR5-evoked phosphorylation of NMDAR in aSyn transgenic (Tg) mice, using the adenosine A_{2A} receptor ($A_{2A}R$) antagonist KW-6002, rescued synaptic and cognitive deficits, supporting the hypothesis that a receptor-mediated mechanism, independent of pore formation and membrane leakage, is sufficient to trigger early synaptic damage induced by extracellular aSyn.

Keywords: α-Synuclein, cellular prion protein, NMDA receptors, metabotropic glutamate 5 receptor, Fyn kinase, synucleinopathies, Parkinson's disease, dementia with Lewy bodies, long term potentiation.

Introduction

The abnormal accumulation of aggregated aSyn in LBs is a common neuropathological hallmark of synucleinopathies such as PD and DLB (Lee et al., 2014; Yang and Yu, 2016). LBs can be detected throughout the brain and are frequently observed in the hippocampus and related brain regions. Synucleinopathies are also characterized by progressive neuronal dysfunction and, eventually, death of affected neuronal populations (Braak and Del Tredici, 2008). In addition to the characteristic motor symptoms of PD, cognitive disturbances also represent an important clinical feature of the disease, and occur not only in advanced stages of the disease, but also in early and even pre-motor phases (Braak et al., 2005; Caviness et al., 2011; Goldman et al., 2014; Kao et al., 2009; Lee et al., 2014; Yang and Yu, 2016).

Recent studies suggest that aSyn oligomers are the most toxic species and that they can be released from neuronal cells, contributing to the major pathological features of synucleinopathies (Lee et al., 2014; Marques and Outeiro, 2012). In fact, extracellular aSyn oligomers, but not monomers or fibers, impair hippocampal LTP - the molecular paradigm involved in learning and memory (Diógenes et al., 2012; Ferreira et al., 2015) - through the activation of NMDAR. This synaptic dysfunction precedes neuronal death, leading to significant changes in resting membrane potential or in input resistance values (Diógenes et al., 2012; Ferreira et al., 2015).

Recently PrP^C was reported to act as a receptor for neurotoxic Aβ oligomers, which share structural and functional similarities with aSyn oligomers (Biasini et al., 2012). PrP^C was additionally suggested to mediate synaptic dysfunction, memory deficits, and neurodegeneration of several β-sheet-rich conformers (Gimbel et al., 2010; Laurén et al., 2009; Resenberger et al., 2011), acting via NMDAR (Larson et al., 2012; Um et

al., 2012). Furthermore, we have previously demonstrated that aSyn oligomers can form complexes with NMDAR at the postsynaptic density (Ferreira et al., 2015), where PrP^C is known to be present (Um et al., 2012).

Here, we found that aSyn interacts physically with PrP^C to mediate Ca^{2+} dysregulation and synaptic dysfunction, through a mechanism involving Fyn phosphorylation and consequent NMDAR2B activation, via mGluR5. Moreover, we found that synaptic and cognitive deficits associated with aSyn overexpression in a Tg mouse model of synucleinopathy are reverted upon blockade of mGluR5-evoked phosphorylation of Src kinases and of the toxic activation of NMDAR2B. Overall, our study sheds light into the early pathophysiological mechanisms preceding aSyn-mediated neurodegeneration and implicates PrP^C as a molecular target in synucleinopathies.

Results

aSyn oligomers impair long-term potentiation through a PrP^C-dependent mechanism

We previously demonstrated that extracellular aSyn oligomers impair LTP in rodent hippocampal slices via a mechanism dependent on NMDAR (Diógenes et al., 2012; Ferreira et al., 2015). PrP^C is known to be involved in NMDAR signaling and, therefore, we hypothesized that PrP^C could mediate the detrimental effects of aSyn oligomers on synaptic plasticity. For this, we compared synaptic function of hippocampal dorsal slices from WT *versus Prnp* null mice (*Prnp*$^{-/-}$) in the presence of extracellular aSyn oligomers (characterized by AFM and SDS-PAGE) (**Supplementary Fig. 3.1a**), as previously described (Diógenes et al., 2012; Ferreira et al., 2015). Synaptic function was assessed by electrophysiological recordings of field excitatory postsynaptic potentials (fEPSPs) in the Schaffer collaterals/CA1 pyramid glutamatergic synapses (**Fig. 3.1a**). As we previously reported (Diógenes et al., 2012; Ferreira et al., 2015), soluble aSyn oligomers significantly decreased the LTP magnitude in hippocampal slices of WT animals (aSyn olig, 500 nM, 90 min; $P < 0.001$; **Fig. 3.1b, c** and **Supplementary Fig. 3.1c, d**), whereas neither aSyn monomers nor fibrils affected LTP magnitude ($P > 0.05$; **Supplementary Fig. 3.1b, c**). Strikingly, in slices from *Prnp*$^{-/-}$ animals, which display normal LTP when compared to WT animals ($P > 0.05$; **Fig. 3.1c, d** and **Supplementary Fig. 3.1d**) (Curtis et al., 2003; Laurén et al., 2009; Lledo et al., 1996), treatment with aSyn oligomers did not affect LTP ($P > 0.05$; **Fig. 3.1d** and **Supplementary Fig. 3.1d)**.

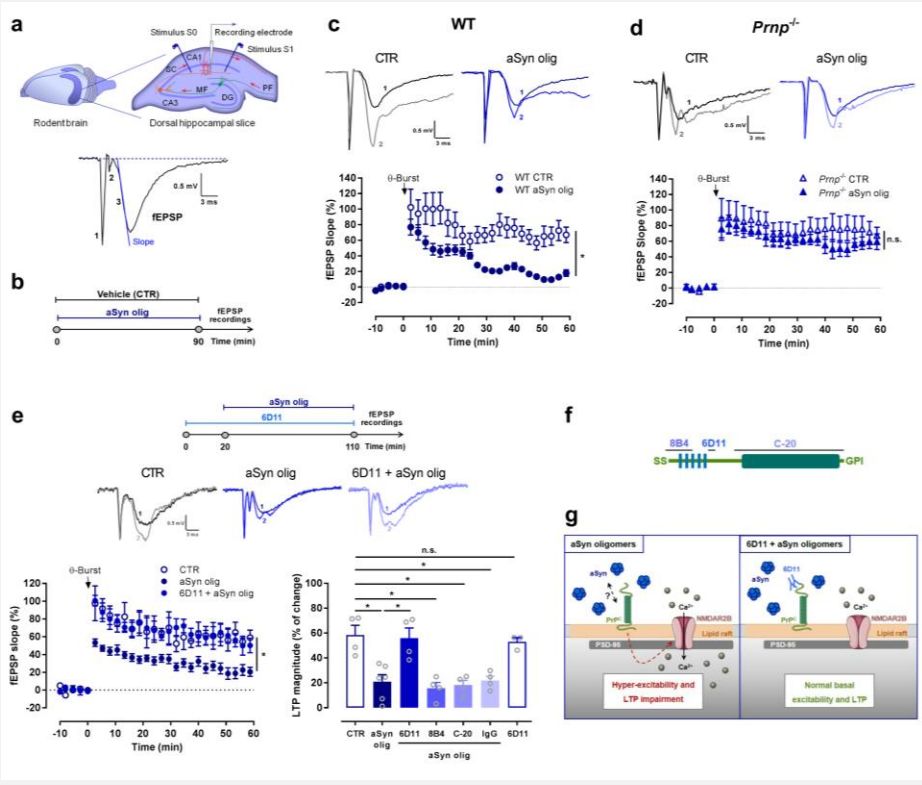


Figure 3.1. PrP^C mediates synaptic impairment induced by extracellular aSyn oligomers. (**a**) Top panel: Schematic representation of the simplified circuitry of the hippocampus. DG: Dentate Gyrus; MF: Mossy Fibers; SC: Schaffer Collaterals; PF: Perforant Pathway; CA3: Cornu Ammonis 3; CA1: Cornu Ammonis 1. A recording electrode and two independent stimulation pathways (S0 and S1), allowing two protocols in the same slice, are placed in the CA1 dendritic area. Bottom panel: a representative field Excitatory Post Synaptic Potential (fEPSP). (1) Stimulus artifact; (2) Fiber volley; (3) fEPSP slope. (**b**) Schematic representation of hippocampal slices incubation protocol. (**c**) Changes in fEPSP slope induced by theta-burst stimulation recorded from WT mice hippocampal slices pre-incubated with extracellular aSyn oligomers (aSyn olig, 90 min, 500 nM, $n = 10$) or in control conditions (CTR, $n = 7$) (means ± s.e.m., $P < 0.001$, one-way ANOVA followed

by a Bonferroni's Multiple Comparison Test). The top panels show representative traces prior (1) and after (2) LTP induction. (**d**) Changes in fEPSP slope were recorded from slices of mice lacking PrP (*Prnp*$^{-/-}$) pre-incubated with extracellular aSyn oligomers (n = 6) or in control conditions (n = 6) (means ± s.e.m., P > 0.05, one-way ANOVA followed by a Bonferroni's Multiple Comparison Test) obtained by the same method as in **c**. (**e**) Left panel: Changes in fEPSP slope were recorded by the same method as in **a**, from hippocampal slices pre-incubated with aSyn oligomers alone (n = 6), in the presence of the 6D11 antibody against PrP^C (6D11, 110 min, 100 nM, n = 4) together with the aSyn oligomers or in control conditions (n = 4). Right panel: Plot of the LTP magnitude represented on the left panel plus the LTP magnitude of WT hippocampal slices in the presence of immunoglobulin G (IgG, 110 min, 100 nM, n = 4) or the anti-PrP^C antibodies, 8B4 (110 min, 10 µM, n = 4) or C-20 (110 min, 10 µM, n = 4) together with aSyn oligomers (change in fEPSP slope at 50–60 min after theta-burst stimulation, compared to baseline) (means ± s.e.m., P < 0.001, one-way ANOVA followed by a Bonferroni's Multiple Comparison Test). Top panels: Schematic representation of hippocampal slices incubation protocol and representative traces prior (1) and after (2) LTP induction. (**f**) Schematic diagram of PrP representing the binding sites of 8B4, 6D11 and C-20 anti- PrP^C antibodies. (**g**) Schematic representation of WT hippocampal synapses exposed to aSyn oligomers alone (left panel) or in the presence of the anti-PrP^C antibody 6D11. 6D11 PrP^C blockade prevents NMDAR overactivation and subunit modification, basal synaptic hyperexcitability, and LTP impairment induced the extracellular aSyn oligomers.

In order to evaluate whether PrP^C was also involved in baseline synaptic efficiency, I/O curves were recorded in slices from WT or *Prnp*$^{-/-}$ animals in the presence or absence of aSyn oligomers. WT slices pre-incubated with aSyn oligomers displayed max slope values higher than control slices ($P < 0.001$; **Supplementary Fig. 3.1e**). This increase in basal excitability was not observed in *Prnp*$^{-/-}$ slices in the presence of aSyn oligomers, which displayed fEPSP slope values that were similar to those in control slices when stimulated with the same intensity ($P > 0.05$; **Supplementary Fig. 3.1f**).

Thus, we concluded that PrP^C is essential for the aSyn oligomer-mediated inhibition of hippocampal LTP.

PrP^C 93-109 amino acid region is required for aSyn oligomer-mediated inhibition of LTP

The absence of sensitivity to aSyn in *Prnp*$^{-/-}$ slices regarding basal excitability and LTP, suggests that PrP^C may act as a key mediator for aSyn synaptic toxicity.

To investigate the region(s) of PrP^C that mediated the aSyn effects, we targeted three regions in the protein, using different antibodies against PrP^C: 6D11 (epitope targeting the region 93-109 of PrP^C; 100 nM), 8B4 (epitope targeting the N-terminus of PrP^C; 10 µg) and C-20 (epitope targeting the C-terminus of PrP^C; 10 µg) (**Fig. 3.1f**). In slices pre-treated with 6D11 anti-PrP^C antibody, the effect of aSyn oligomers on LTP was blocked ($P < 0.001$; **Fig. 3.1e**). In contrast, pre-treatment with 8B4 or C-20 had no effect ($P > 0.05$; **Fig. 3.1e** and **Supplementary Fig. 3.1h**). This suggests that the 93-109 segment of PrP^C is the crucial region for aSyn-induced toxic effects. Accordingly, the 6D11 anti-PrP^C antibody also prevented the effect of aSyn oligomers on the I/O curve ($P > 0.05$; **Supplementary Fig. 3.1g**). Presynaptic short-term plasticity was not

altered by aSyn oligomers nor by the 6D11 antibody under these conditions, as evaluated by paired-pulse facilitation (PPF) ($P > 0.05$; **Supplementary Fig. 3.1i**). We also confirmed that 6D11 alone did not affect LTP, nor did IgG modify the aSyn-mediated reduction of LTP (**Fig. 3.1e,** bar graph).

Thus, PrP^C deletion or blockade at the 93-109 segment prevents aSyn oligomer-induced impairments both in LTP and basal synaptic transmission, suggesting that PrP^C mediates this synaptic dysfunction in a 6D11-sensitive manner (**Fig. 3.1g**).

PrP^C modulates aSyn-mediated synaptic impairment through the activation of Src Tyr-kinases and NMDAR2B

The function of PrP^C relates to the modulation of phosphorylation cascades, in particular, the one governed by Fyn (Linden et al., 2008; Sorgato and Bertoli, 2009; Wulf et al., 2017), a member of the Src Tyr-kinase family (SFK) that is highly expressed in neurons (Um and Strittmatter, 2013). Moreover, both Fyn and PrP^C localize in lipid rafts, and clustering of PrP^C activates Fyn in cell lines (Mouillet-Richard et al., 2007; Pantera et al., 2009; Williamson et al., 2007). Fyn was also reported to co-localize with PrP^C at the postsynaptic density (PSD), where it plays a critical role in cognition and LTP by mediating NMDAR phosphorylation and, consequently, excitotoxicity (Grant et al., 1992; Nakada et al., 2011; Suzuki and Okumura-Noji, 1995; Weilinger et al., 2016). As such, the tyrosine kinase Fyn is a candidate mediator of signal transduction from an aSyn/PrP^C interaction. We assessed the involvement of Fyn in aSyn-associated synaptic deficits. For this we treated rat hippocampal slices with aSyn oligomers alone or in the presence of a selective SFK inhibitor, 1-Naphtlyl PP1 (PP1, 30 µM, 110 min; **Fig. 3.2a**), and induced LTP as before. As expected, the LTP magnitude was

significantly reduced in slices pre-incubated with aSyn oligomers ($P < 0.01$; **Fig. 3.2a** and **Supplementary Fig. 3.2a**). When Fyn was blocked by PP1, the LTP magnitude was reestablished to control values ($P < 0.01$; **Fig. 3.2a** and **Supplementary Fig. 3.2a**). PP1 alone did not affect the LTP magnitude ($P > 0.05$; **Supplementary Fig. 3.2a**). The aSyn-induced shift in I/O curve was also lost when Fyn activation was inhibited by PP1 (**Supplementary Fig. 3.2b**).

Next, we hypothesized that PrP^C/Fyn activation converged to activate NMDAR (Nakazawa et al., 2001; Salter and Kalia, 2004). To test this, we evaluated the effect of the NMDAR antagonist APV (50 μM) on basal synaptic transmission. APV did not modify the fEPSP slope in control WT slices ($P > 0.05$; **Fig. 3.2b** and **Supplementary Fig. 3.2c**). In contrast, the acute application of APV induced a progressive reduction of the fEPSP in aSyn oligomer-treated WT slices ($P < 0.001$; **Fig. 3.2b** and **Supplementary Fig. 3.2c**), indicating a basal activation of NMDAR caused by aSyn (Diógenes et al., 2012; Ferreira et al., 2015). Inhibition of Fyn by PP1 prevented this effect ($P < 0.01$; **Fig. 3.2b** and **Supplementary Fig. 3.2c**). This loss of aSyn effect was also observed in slices from *Prnp*$^{-/-}$ mice, compared to their WT littermates (**Fig. 3.2c** and **Supplementary Fig. 3.2d, e**). We showed previously that exposure to aSyn is associated with an increase in NMDAR subunit 2B (NMDAR2B) levels (Ferreira et al., 2015). We now found that, in *Prnp*$^{-/-}$ mice, aSyn oligomers lost the ability to alter NMDAR2B levels, whereas the levels of NMDAR subunit 1 (NMDAR1) were not altered by aSyn in WT or *Prnp*$^{-/-}$ mice (**Supplementary Fig. 3.2f**).

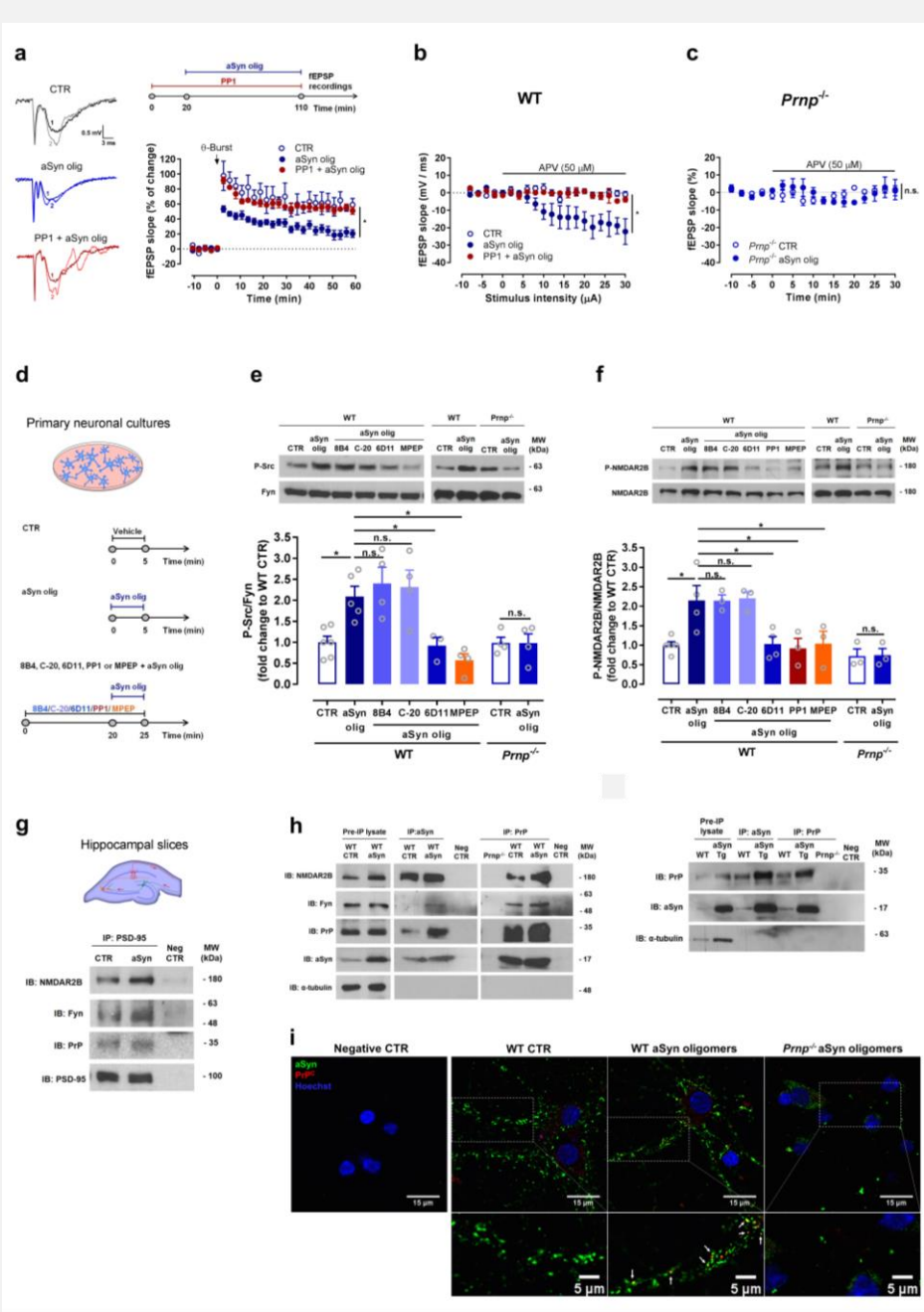


Figure 3.2. PrP^C dependent-toxic effects of aSyn oligomers are mediated by Src family-kinases. (**a**) Top panel: schematic representation of hippocampal slices incubation protocol. Bottom panels: Representative traces prior (1) and after (2) LTP induction and changes in fEPSP slope in hippocampal slices from WT rat in control conditions (CTR, *n* = 4), and pre-incubated with aSyn oligomers alone (aSyn olig, 90 min, 500 nM, *n* = 6) or in

the presence of the Src-family inhibitor, 1-naphthyl PP1 (PP1, 110 min, 30 µM; PP1 + aSyn olig, n = 3; means ± s.e.m., P < 0.01, one-way ANOVA followed by a Bonferroni's Multiple Comparison Test). (**b**) Effect of the NMDAR antagonist APV (50 µM, 30 min) superfusion on basal fEPSP slope from control WT hippocampal slices in the same conditions as in **a** (means ± s.e.m., P < 0.01, one-way ANOVA followed by a Bonferroni's Multiple Comparison Test). (**c**) Effect of APV superfusion on basal fEPSP slope in *Prnp*$^{-/-}$ hippocampal slices in the same conditions as in **b** (P > 0.05). (**d**) Schematic representation of primary neuronal cultures incubation protocol. (**e**) Representative immunoblots and quantification of the phospho-Src levels, normalized to Fyn immunoreactivity, in WT and *Prnp*$^{-/-}$ primary neuronal cultures in control conditions (n = 6), and treated with aSyn oligomers alone (n = 5) or in the presence of the 6D11, C-20, 8B4 antibodies against PrP^C (n = 3-4) or the selective mGluR5 antagonist (MPEP, n = 4; means ± s.e.m., P < 0.001, one-way ANOVA followed by a Bonferroni's Multiple Comparison Test). (**f**) Representative immunoblots and quantification of the phospho-NMDAR2B levels, normalized to NMDAR immunoreactivity, in the same conditions as in **e** plus in the presence of 1-naphthyl-PP1 (n = 3; means ± s.e.m., P < 0.001, one-way ANOVA followed by a Bonferroni's Multiple Comparison Test). (**g**) Representative western blot of 3 independent experiments showing immunoprecipitation of PSD-95 in WT hippocampal control slices and slices pre-incubated with aSyn oligomers. Membranes were immunoblotted with anti-NMDAR2B, anti-Fyn, anti-PrP and anti-PSD-95 antibodies. (**h**) Representative western blot of 3 independent experiments showing immunoprecipitation of aSyn and PrP, in aSyn Tg, *Prnp*$^{-/-}$, and WT hippocampal slices in control conditions and pre-incubated with aSyn oligomers. Membranes were immunoblotted with anti-NMDAR2B, anti-Fyn, anti-PrP^C and anti-aSyn antibodies. IgG was used as a negative control (Neg CTR). (**i**) Immunohistochemistry of WT and *Prnp*$^{-/-}$ primary neuronal cultures (representative image of n = 3). aSyn is labelled in green, PrP^C is labelled in red, and cell *nuclei* are stained with Hoechst in blue (scale bar: 15 µm). At the bottom, a 63x magnification image (scale bar: 5 µm). Colocalization is

indicated by arrows and was assessed by Mander's overlap coefficient method using ZEN Software (Zeiss). PrP^C-bound aSyn was detected in WT neuronal cultures pre-treated with aSyn oligomers for 5 min (Mander's overlap coefficient = 0.70), compared to control conditions, where no colocalization was observed (Mander's overlap coefficient = 0). No surface binding of aSyn oligomers was detected in *Prnp*$^{-/-}$ cultures (Mander's overlap coefficient = 0).

We then evaluated Fyn activation by quantifying Src phosphorylation levels. We exposed primary neuronal cultures (12 DIV) to aSyn oligomers for different time periods. At this stage, neurons were fully mature, as confirmed by morphological analysis and MAP2 staining (**Supplementary Fig. 3.3a**). We detected a maximal level of phospho-Src after a 5-min exposure, with no alterations in Fyn (**Supplementary Fig. 3.3c, d**). This was coincident with a significant increase in aSyn levels (**Supplementary Fig. 3.3b**). We asked whether PrP^C was required for the observed aSyn-induced Src activation, but we could not detect any effect of aSyn on Src activation in neuronal cultures from *Prnp*$^{-/-}$ mice ($P > 0.05$; **Fig. 3.2e**). Additionally, we found that the 6D11-PrP^C antibody prevented Src activation by aSyn ($P < 0.001$; **Fig. 3.2d, e**), whereas neither 8B4 nor C-20 antibodies prevented Src phosphorylation. These effects on Src activation were not due to alterations in PrP^C levels since similar levels of PrP^C were detectable after aSyn exposure for different time periods (**Supplementary Fig. 3.3e**).

NMDAR plays a key role in synaptic plasticity and in aSyn-induced LTP impairment (Diógenes et al., 2012). Intracellular segments of NMDAR2A and NMDAR2B subunits are phosphorylated on tyrosine residues by SFK (Salter and Kalia, 2004). Of these, Y1472 of NMDAR2B is a major phosphorylation site of Fyn kinase (Nakazawa et al., 2001). We examined total and phosphorylated levels of Y1472 NMDAR2B in neuronal cultures exposed to aSyn oligomers for different time periods. At

5 min of exposure, an increase in the levels of phospho-NMDAR2B was already detected, with no changes in the total levels of NMDAR2B (**Supplementary Fig. 3.3f, g**). This increase was blocked by the 6D11 anti-PrP^C and the Fyn inhibitor (PP1, 30 µM, 25 min; $P < 0.05$; **Fig. 3.2f**), but was not affected by 8B4 or C-20 anti-PrP^C antibodies. In agreement, aSyn oligomers failed to induce further NMDAR2B phosphorylation in neuronal cultures from *Prnp*$^{-/-}$ mice (**Fig. 3.2f**). Thus, aSyn requires PrP^C, in particular, the 93-109 region, in order to induce Fyn activation and subsequent NMDAR2B phosphorylation, which may underlie the LTP impairments observed in the presence of aSyn oligomers.

aSyn physically interacts with PrP^C to form a complex with NMDAR2B and Fyn kinase at the postsynaptic membrane

In our previous studies reported in Chapter II, we demonstrated a postsynaptic action of aSyn oligomers in hippocampal synapses, where aSyn forms a complex with NMDAR2B (Ferreira et al., 2015). Proteomic analyses revealed PrP^C is a component of the postsynaptic density (PSD) (Collins et al., 2006). Consistently, PrP^C co-fractionates with PSD-95 and Fyn kinase, and is involved in Fyn activation in cell lines and in animal models (Mouillet-Richard et al., 2007; Pantera et al., 2009; Williamson et al., 2007).

To test whether aSyn interacted with PrP^C, we first showed that the PrP^C/Fyn/NMDAR2B complex is increased after exposure to aSyn oligomers, by co-immunoprecipitation (co-IP) of PSD-95 (**Fig. 3.2g**). Next, by pulling down PrP^C we detected Fyn, NMDAR2B, and aSyn in the same complex in WT, but not in *Prnp*$^{-/-}$ slices, both in CTR or aSyn exposed slices **(Fig. 3.2h)**. This was further validated by the reverse co-IP, where we pulled down aSyn and detected NMDAR2B, Fyn, and PrP^C (**Fig. 3.2h**). As expected, a non-interacting protein, α-tubulin, was only detected

in the pre-IP lysates, with no detectable signal in neither aSyn nor PrP^C IPs, further confirming the specificity of aSyn-PrP^C association. In all these conditions, co-IP with IgG (negative control) did not yield any bands (**Fig. 3.2h**). In addition, we detected PrP^C-bound aSyn in WT neuronal cultures pre-treated with aSyn oligomers (**Fig. 3.2i**). In control conditions, no co-localization was observed. Moreover, we did not detect any surface binding of aSyn oligomers in *Prnp*$^{-/-}$ cultures (**Fig. 3.2i**).

To confirm the aSyn-PrP^C interaction *in vivo*, we used Tg mice expressing human WT aSyn under control of Thy1 promoter (Thy1-aSyn mice), which display significant levels of aSyn in the hippocampus prior to nigrostriatal modifications (Chesselet et al., 2012; Magen et al., 2012). When we immunoprecipitated either PrP^C or aSyn from the hippocampus of aSyn Tg mice, we detected the counterpart of the aSyn-PrP^C complex (**Fig. 3.2h)**. Moreover, we found that the PrP^C levels followed the increase in aSyn in the hippocampus (**Supplementary Fig. 3.5a, e**), as reported before for Aβ oligomers (Caetano et al., 2011).

Taken together, our data confirm an association of aSyn with PrP^C and the formation of a PrP^C-Fyn-NMDAR2B protein complex at the post-synaptic density.

aSyn oligomers impair calcium homeostasis through a PrP^C-dependent mechanism

Phosphorylation of NMDAR2B mediates alterations in NMDAR-induced calcium levels. To investigate the involvement of calcium signaling disruption as a consequence of aSyn oligomer-mediated NMDAR hyperactivation we measured variations in intracellular calcium concentrations ($[Ca^{2+}]i$) in primary neuronal cultures. Changes in $[Ca^{2+}]i$ were detected by Ca^{2+} imaging using fura 2-acetoxymethyl ester (Fura 2AM) (**Fig. 3.3a**).

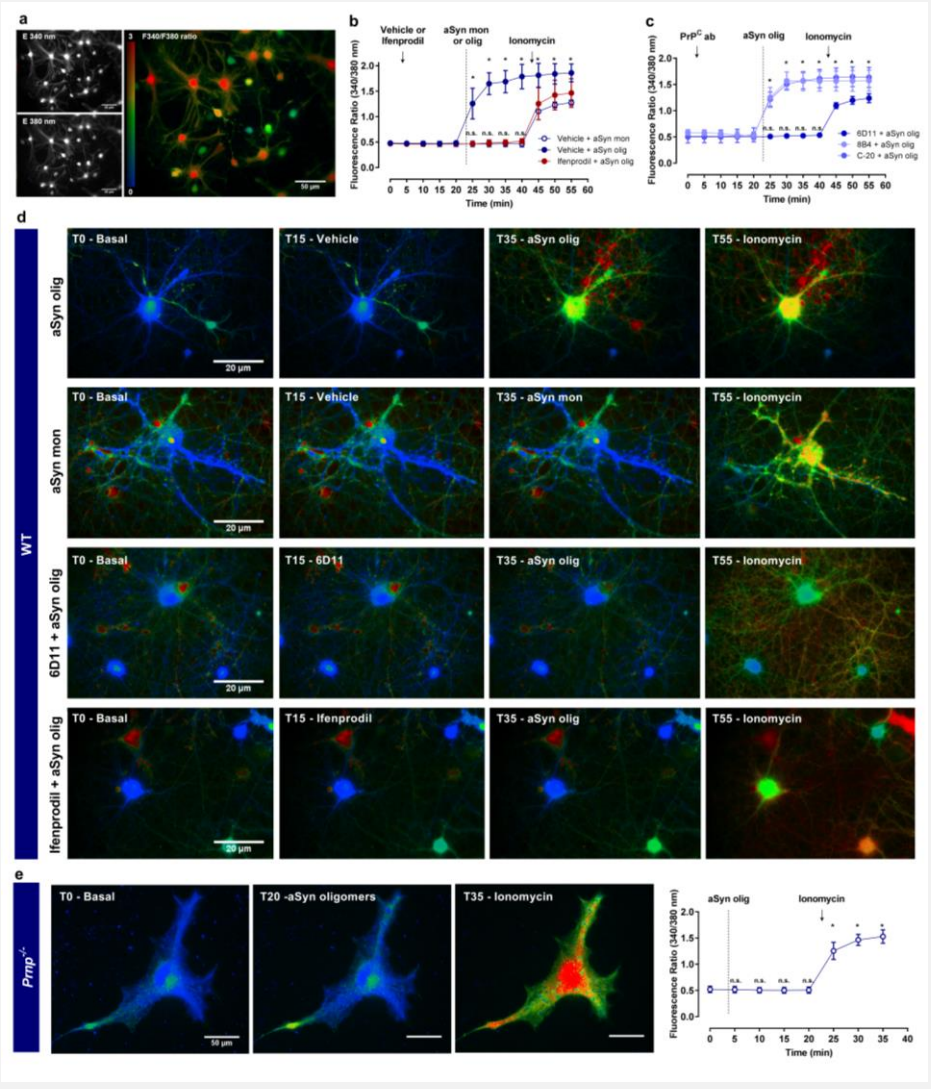


Figure 3.3. aSyn oligomers, but not monomers, increase intracellular Ca^{2+} levels in primary neuronal cultures in a PrP^C/NMDAR2B-dependent mechanism. (**a**) Representative images of Ca^{2+} imaging. Bright regions indicate the location of cytoplasm and organelles, where the concentration of Ca^{2+} is higher than in the dark regions indicating the intercellular medium, where diffusion processes take place. Right image corresponds to the ratio between the radiation emitted at 510 nm, when cells are excited at 340 nm, over emission upon excitation at 380 nm (F340/F380). (**b, c**) Graphs showing a 55 min time course of Ca^{2+}-dependent fluorescence recorded and averaged

from FURA-2 AM WT neurons in response to aSyn monomers (aSyn mon, 20min, 500 nM), oligomers (aSyn olig, 20 min, 500 nM), and oligomers in the presence of selective NMDAR2B antagonist, Ifenprodil (40 min, 3 µM) or in the presence of the 6D11 (40 min, 100 nM), 8B4 (40 min, 10 µM), or C-20 (40 min, 10 µM) antibodies against PrP. (**d**) WT representative images of the different conditions showed in **b**, **c**. (**e**) *Prnp*$^{-/-}$ representative images and graphs showing a 55 min time course of Ca^{2+}-dependent fluorescence recorded and averaged from FURA-2 AM *Prnp*$^{-/-}$ neurons before and after exposure to aSyn oligomers (20 min, 500 nM). Cells were challenged with ionomycin (15 min, 2 µM) at the conclusion of each experiment. Each point represents the means ± s.e.m. of 340/380 nm readings of 20-25 responsive cells *per* experimental condition from 3 independent cultures. $P < 0.001$, one-way ANOVA followed by a Bonferroni's Multiple Comparison Test.

Application of oligomeric forms of aSyn rapidly elevated intracellular Ca^{2+} levels (**Supplementary Video 1**), whereas equivalent amounts of aSyn monomers evoked no detectable changes in fluorescence ($P < 0.001$; **Fig. 3.3b, d**). This aSyn oligomer-evoked increase in $[Ca^{2+}]i$ was prevented by the 6D11 PrP antibody ($P < 0.001$; **Fig. 3.3c, d**), but not by either C-20 or 8B4 antibodies (**Fig. 3.3c, d**). None of the antibodies alone affected calcium levels (**Fig. 3.3c, d**). When the specific NMDAR2B subunit was blocked by ifenprodil (3 µM), the effect of aSyn oligomers on $[Ca^{2+}]i$ was also suppressed ($P < 0.001$; **Fig. 3.3b, d**). More importantly, in primary neuronal cultures of *Prnp*$^{-/-}$ mice, aSyn oligomers failed to induce any increase in intracellular Ca^{2+} levels (**Fig. 3.3e** and **Supplementary Video 2**), showing that the aSyn-mediated calcium deregulation depends on PrP^{C}/NMDAR2B-mediated signaling.

mGluR5 mediates aSyn/PrP^C synaptic dysfunction

Although PrP^C and SFK are enriched in the postsynaptic density (PSD) (Collins et al., 2006; Um et al., 2012), the connection of aSyn/PrP^C to SFK cannot be direct, since PrP^C is anchored via glycolipid to the plasma membrane, whereas SFK kinases are cytoplasmic (Salter and Kalia, 2004). Previous studies reported metabotropic glutamate receptor 5 (mGluR5) as a mediator of Fyn activation promoted by PrP^C (Um and Strittmatter, 2013). We hypothesized that mGluR5, a PSD transmembrane protein (Emes et al., 2008; Um et al., 2013), could mediate aSyn/PrP^C signaling leading to impaired neuronal function. First, we treated primary cultures with the selective mGluR5 antagonist MPEP (5 μM), prior to a 5 min exposure to aSyn oligomers. Blockade of mGluR5 prevented both Fyn and NMDAR2B activation, measured by Src and NMDAR2B phosphorylation levels. To investigate the functional role of this mGluR5-mediated phosphorylation of NR2B subunit, we treated rat hippocampal slices with aSyn oligomers in the presence of MPEP (5 μM, 110 min; **Fig. 3.4a**) and induced LTP in Schaffer collaterals/CA1 pyramid glutamatergic synapses by theta-burst stimulation. Interestingly, mGluR5 blockade by MPEP prevented LTP impairment induced by aSyn oligomers alone ($P < 0.001$; **Fig. 3.4b** and **Supplementary Fig. 3.4a**). MPEP alone did not affect the LTP magnitude ($P > 0.05$; **Supplementary Fig. 3.4a**). Furthermore, when mGluR5 was activated by the selective agonist DHPG (10 μM, 110 min; **Fig. 3.4c**), the protective effect of the anti-PrP^C antibody 6D11 against aSyn-induced LTP impairment, was no longer detectable ($P < 0.001$; **Fig. 3.4d** and **Supplementary Fig. 3.4c**). DHPG alone did not change the effects of aSyn on LTP ($P > 0.05$; **Supplementary Fig. 3.4c**). Taken together, these data suggest that mGluR5 acts downstream of PrP^C and probably serves as a bridge between PrP^C and SFK/NMDAR2B to impair synaptic function.

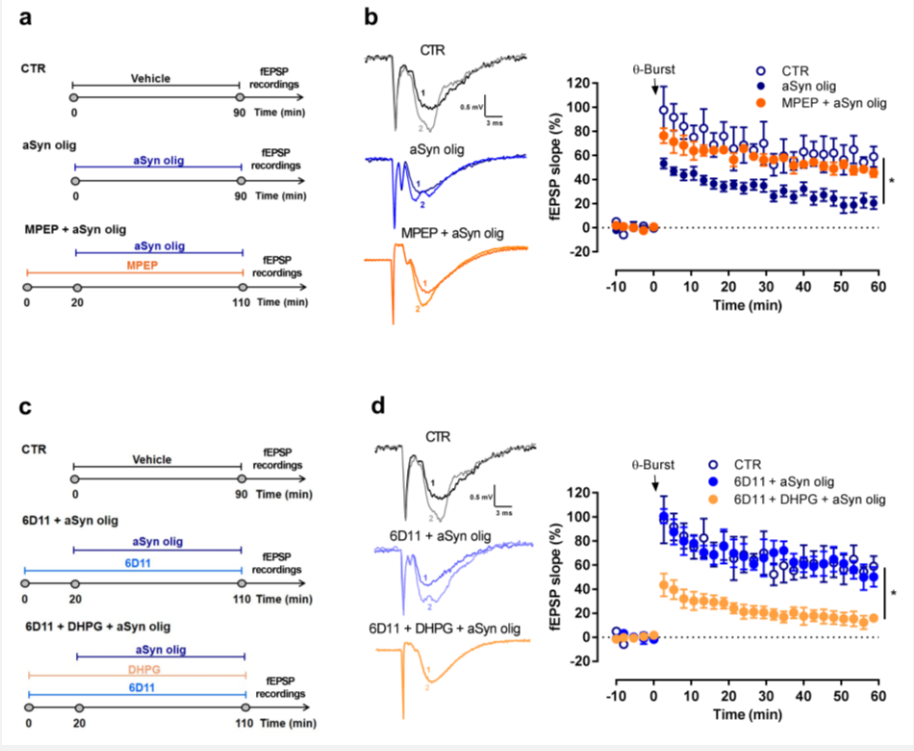


Figure 3.4. PrP^C blockade rescues LTP impairment induced by extracellular aSyn oligomers through a mechanism dependent on mGluR5. (**a**) Schematic representation of hippocampal slices incubation protocol used in **b**. (**b**) Changes in fEPSP slope induced by theta-burst stimulation recorded from WT hippocampal slices in control conditions (CTR, n = 4), pre-incubated with extracellular aSyn oligomers alone (aSyn olig, 90 min, 500 nM, n = 6) or in the presence of the mGluR5-selective antagonist MPEP (110 min, 5 µM; MPEP + aSyn olig, n = 4; means ± s.e.m., $P < 0.001$, one-way ANOVA followed by a Bonferroni's Multiple Comparison Test). The left panels show representative traces prior (1) and after (2) LTP induction. (**c**) Schematic representation of hippocampal slices incubation protocol used in **d**. (**d**) Changes in fEPSP slope induced by theta-burst stimulation recorded from WT hippocampal slices in control conditions (n = 4), pre-incubated with extracellular aSyn oligomers together with the 6D11 antibody (110 min, 100 nM; 6D11 + aSyn olig, n = 4) and in the presence of the mGluR5-selective agonist DHPG (110 min, 10 µM; 6D11 + DHPG + aSyn

olig, $n = 4$; means ± s.e.m., $P < 0.001$, one-way ANOVA followed by a Bonferroni's Multiple Comparison Test). The left panels show representative traces prior (1) and after (2) LTP induction.

Blockade of mGluR5-evoked tyrosine phosphorylation of NMDAR2B reverses memory deficits in a Tg mouse model of PD

Phosphorylation of the NMDAR2B-Y1472 residue by Fyn kinase is under tight regulation of mGluR5 via adenosine A_{2A} receptors ($A_{2A}R$) (**Fig. 3.7**). Blockade of $A_{2A}R$ effectively inhibits NMDAR2B phosphorylation evoked by mGluR5 (Sarantis et al., 2015) and prevents LTP impairment and NMDAR overactivation mediated by aSyn *in* vitro as discussed in Chapter II (Ferreira et al., 2015). Accordingly, we observed that $A_{2A}R$ blockade prevented aSyn-mediated Src kinase phosphorylation in primary cultures (**Fig. 3.6h**). Furthermore, blocking $A_{2A}R$ (SCH-58261, 50 nM) in hippocampal slices protected against aSyn-mediated LTP impairments (**Supplementary Fig. 3.4b**), and this protective effect was lost if mGluR5 were activated, reinforcing a common signaling pathway for the effects observed ($P > 0.05$; **Supplementary Fig. 3.4b, c**).

To provide *in vivo* evidence for this mechanism, we assessed the effect of an $A_{2A}R$ blocker (KW-6002, also known as istradefylline) (Batalha et al., 2013, 2016; Coelho et al., 2014), particularly suited for chronic administration based on its bioavailability and brain penetration (Yang et al., 2007), to rescue memory and synaptic impairments in aSyn Tg mice (Thy1-aSyn). These animals express human aSyn in the whole hippocampus (**Supplementary Fig. 3.5a**), with an enrichment in cell bodies and axons of pyramidal neurons, in comparison to WT mice, in which the mouse aSyn mainly co-localizes with the presynaptic marker, SNAP25 (**Supplementary Fig. 3.5b**). Five-month-old aSyn Tg mice and their WT littermates were treated for one month with KW-6002, delivered

in the drinking water, at a dose known to be effective *in vivo* (3 mg/Kg/day) (Batalha et al., 2013; Coelho et al., 2014), (as depicted in **Fig. 3.5a**).

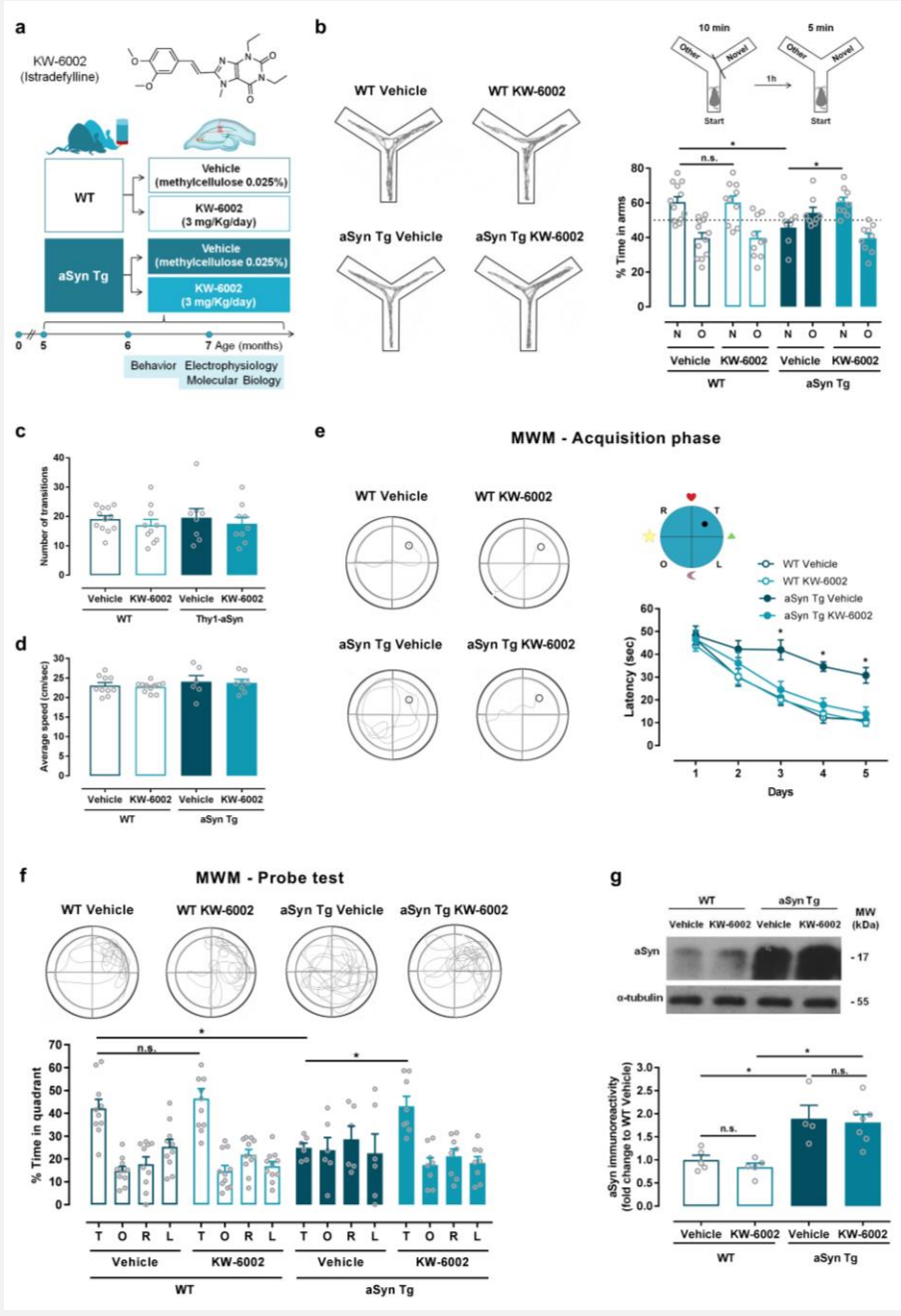


Figure 3.5. ***In vivo*** **treatment of Thy1-aSyn (aSyn Tg) mice with KW-6002 rescues aSyn-associated cognitive deficits.** (**a**) Schematic representation of the groups of animals used and the corresponding oral pharmacological

treatment (vehicle or KW-6002). (**b**) Spatial memory performance was assessed by the Y-Maze test. Schematic representation of the Y-maze test (top panel). Representative traces (left panel) and quantification of the time spent in novel arm (N) *versus* the other arm (O) in the different groups of animals represented in **a** (n = 8-12; means ± s.e.m., $P < 0.05$, two-way ANOVA followed by a Tukey post-hoc comparison test). (**c**) Quantification of the number of transitions between arms observed in each group (n = 8-12; means ± s.e.m., $P > 0.05$, two-way ANOVA followed by a Tukey post-hoc comparison test). (**d**) Quantification of the average swimming speed during Morris water maze (MWM) probe test (n = 6-11; means ± s.e.m., $P > 0.05$, two-way ANOVA followed by a Tukey post-hoc comparison test). Hippocampal-dependent memory performance was assessed by the MWM test, in which acquisition (**e**) and retention (**f**) were evaluated (n = 6-11; means ± s.e.m., $P < 0.05$, two-way ANOVA followed by a Tukey post-hoc comparison test). (**g**) Representative immunoblots and quantification of aSyn levels in the hippocampus of WT and aSyn Tg mice treated with vehicle (n = 5 and 4) or KW-6002 (n = 5 and 7) (means ± s.e.m., $P < 0.001$, two-way ANOVA followed by a Tukey post-hoc comparison test). α-Tubulin was used as a loading control.

Hippocampal-dependent memory was assessed by the Morris Water Maze (MWM) test, in which memory acquisition and retention were evaluated. Chronic blockade of $A_{2A}R$ completely reverted the learning and memory deficits induced by aSyn overexpression. During the acquisition phase, treatment of aSyn Tg with KW-6002 restored learning ($P < 0.001$; **Fig. 3.5e**), when compared to animals treated with vehicle, which exhibited a slower learning performance to find the hidden platform, showing deficits at day 3, 4 and 5 ($P < 0.001$; **Fig. 3.5e**). Furthermore, KW-6002 treatment reestablished the retention ability of aSyn Tg mice, as observed in the probe test, measured by the time spent in the target

quadrant compared to the other quadrants (**Fig. 3.5f**). No changes were observed in the overall swimming speed among groups (**Fig. 3.5d**).

Short-term reference memory was assessed in a spontaneous novelty-based spatial preference Y-maze test. aSyn Tg mice performed worse than WT mice, revealing no preference for the novel arm ($P < 0.05$; **Fig. 3.5b**). Importantly, KW-6002 restored memory impairments in aSyn Tg animals, as observed by the increased time spent in the novel arm ($P < 0.05$; **Fig. 3.5b**). No changes were observed in the number of transitions between arms ($P > 0.05$; **Fig. 3.5c**), discarding significant motor impairments as described, since at this early stage these aSyn Tg mice exhibit mostly cognitive and memory deficits, prior to nigrostriatal pathology (Chesselet et al., 2012). This correlated with the absence of significant dopaminergic neuronal loss, evaluated by tyrosine hydroxylase (TH) levels (**Supplementary Fig. 3.5c, d**). Furthermore, we did not detect any influence of the treatment with KW-6002 on the levels of aSyn in the hippocampus ($P > 0.05$; **Fig. 3.5g**).

Treatment with KW-6002 also rescued LTP deficits induced by aSyn overexpression, without affecting LTP magnitude in WT animals ($P < 0.05$; **Fig. 3.6a, b**). Moreover, the treatment also rescued the I/O alterations observed in aSyn Tg mice, without changing the I/O curve in WT mice ($P < 0.001$; **Fig. 3.6c**). No changes were observed in the magnitude of long-term depression (LTD) or in PPF across genotypes or treatment ($P > 0.05$; **Fig. 3.6d, e, f**).

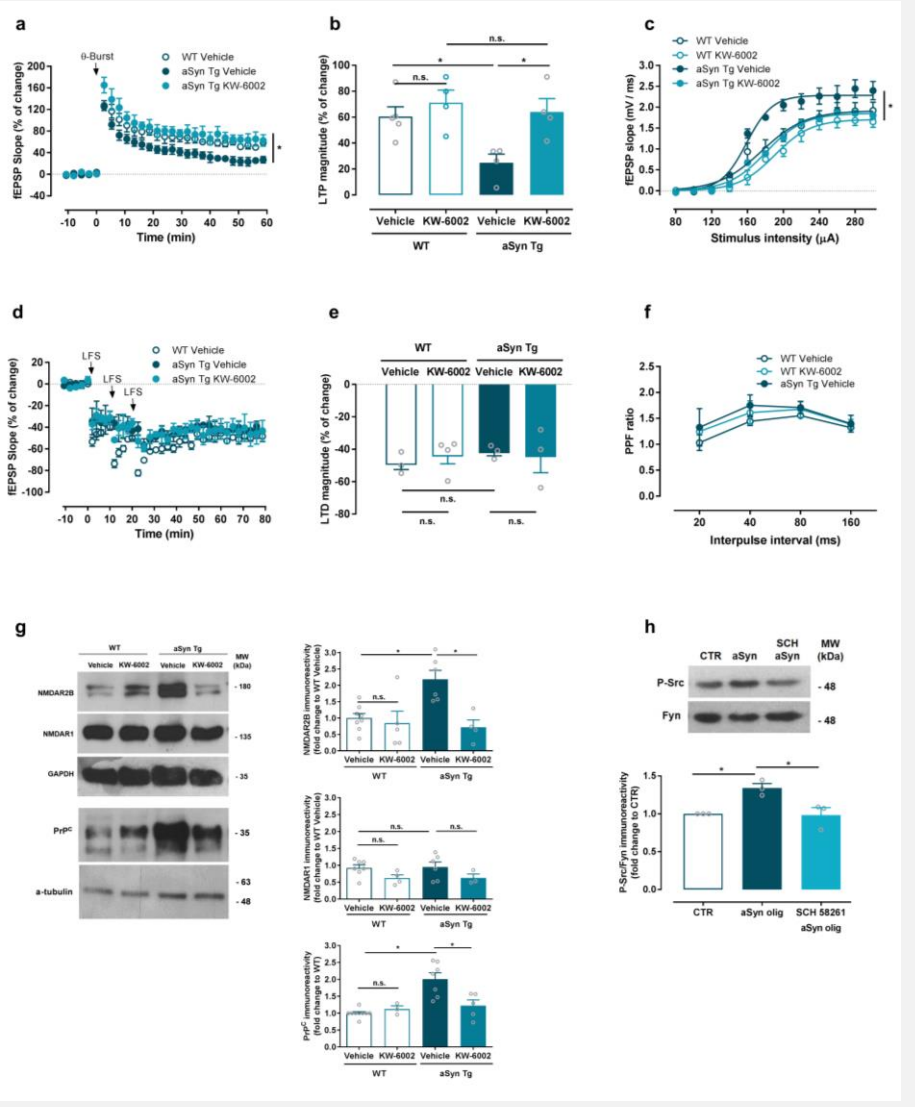


Figure 3.6. aSyn-associated synaptic and NMDA receptor dysfunction are rescued by KW-6002 *in vivo* treatment. (**a**) Changes in the fEPSP slope upon LTP induced by theta-burst stimulation from hippocampal slices. (**b**) LTP magnitude after theta-burst stimulation (change in fEPSP slope at 50–60 min) (n = 4-5; means ± s.e.m., $P < 0.05$, two-way ANOVA followed by a Tukey post-hoc comparison test). (**c**) Input/output (I/O) curves corresponding to fEPSP slope evoked by different stimulation intensities (60–300 µA) (n = 3-5; means ± s.e.m., $P < 0.001$, F-test). (**d**) Changes in fEPSP slope upon LTD induction (theta-burst stimulation) obtained for WT and Thy1-aSyn (aSyn Tg)

mice treated with vehicle or KW-6002. (**e**) LTD magnitude after theta-burst stimulation (change in fEPSP slope at 50–60 min) (*n* = 3-4; means ± s.e.m., *P* > 0.05, two-way ANOVA followed by a Tukey post-hoc comparison test). (**f**) PPF plotted against different interpulse intervals in WT and aSyn Tg mice treated with vehicle or KW-6002 (*n* = 3; means ± s.e.m., *P* > 0.05, two-way ANOVA followed by a Tukey post-hoc comparison test). (**g**) Representative immunoblot and quantification of NMDA receptor subunit 2B (NMDAR2B) and NMDA receptor subunit 1 (NMDAR1) levels in WT and aSyn Tg mice hippocampus in the same conditions as shown in **a** (*n* =4-8; means ± s.e.m., *P* < 0.01, two-way ANOVA followed by a Tukey post-hoc comparison test). GAPDH was used as a loading control. (**h**) Representative immunoblots and quantification of the phospho-Src levels, normalized to Fyn immunoreactivity, in WT and *Prnp*$^{-/-}$ primary neuronal cultures in control conditions (CTR, *n* = 3), and treated with aSyn oligomers alone (aSyn olig, *n* = 3) or in the presence of the SCH-58261 (SCH, 50 nM, *n* = 3) (means ± s.e.m., *P* < 0.05, one-way ANOVA followed by a Bonferroni's Multiple Comparison Test).

Finally, we found that the aSyn Tg mice had increased levels of NMDAR2B in the hippocampus when compared to WT littermates. Selectively blocking mGluR5-Fyn signaling with KW-6002 treatment restored the levels of NMDAR2B in the aSyn Tg animals (**Fig. 3.6g**), providing evidence that this pathway regulates the aSyn-mediated effects *in vivo* (**Fig. 3.7**).

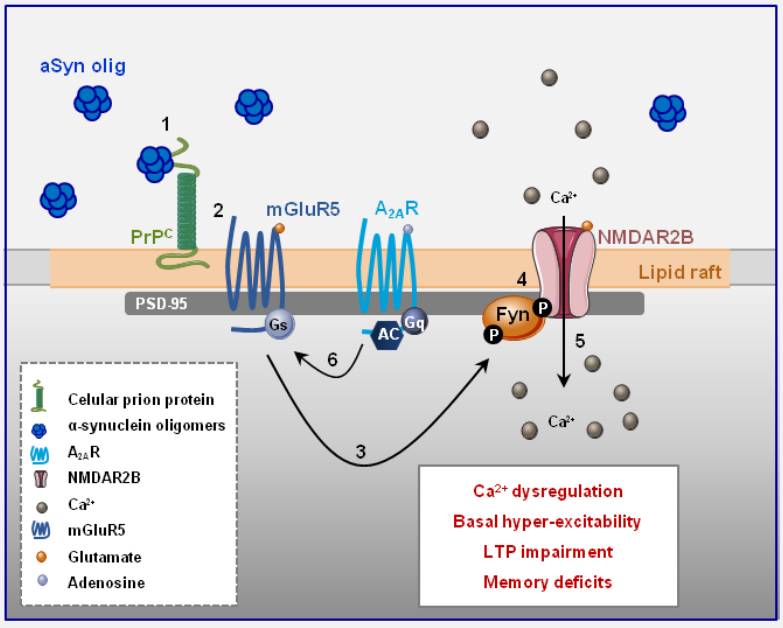


Figure 3.7. Summary diagram of the mechanism by each aSyn oligomers induce an aberrant PrP^C-mGluR5-NMDAR2B signaling at the post-synaptic density. aSyn oligomers physically interact with PrP^C (**1**) to activate mGluR5 (**2**) (Di Scala et al., 2016a). This leads to the phosphorylation and activation of the Src kinase Fyn (**3**) followed by the phosphorylation of NMDAR2B (Y1472) (**4**) (Yang et al., 2007) and robust increase in intracellular Ca^{2+} (**5**). Adenosine $A_{2A}R$ are required for the mGluR5-induced NMDAR2B phosphorylation (**6**) (Urrea et al., 2017).

Discussion

The main finding of this chapter is that aSyn oligomeric species interact with PrP^C through mGluR5, activating SFK kinases and, subsequently, NMDAR2B. We discovered a physical interaction between aSyn, PrP^C, NMDAR2B and Fyn kinase in the post synaptic density (PSD) of the hippocampus, supporting a role of this interaction in the pathophysiological effects induced by aSyn. Furthermore, we show for the first time that genetic or antibody-mediated inactivation of PrP^C prevent the toxic effects of aSyn on synaptic function. This protective effect afforded by PrP^C inhibition is due to prevention of aberrant SFK/NMDAR2B signaling triggered by mGluR5, and the reestablishment of intracellular Ca^{2+} homeostasis. Finally, we confirmed the importance of this mechanism *in vivo,* in a mouse model of synucleinopathies based on the overexpression of human aSyn, by rescuing synaptic and cognitive deficits upon $A_{2A}R$ blockade and consequently inhibition of mGluR5-evoked phosphorylation of NMDAR. Together, these data support the hypothesis that a receptor-mediated mechanism, independent of pore formation and membrane leakage, is sufficient to trigger early synaptic damage induced by extracellular aSyn, which could occur both as part of the normal biology of the protein or during the spreading of pathology in PD and other synucleinopathies.

aSyn aggregation, synaptic dysfunction and consequent neuronal cell loss are key neuropathological hallmarks of synucleinopathies, but the precise molecular mechanisms and nature of the toxic species produced during aggregation remained unclear (Schulz-Schaeffer, 2010). Nevertheless, extracellular soluble aSyn oligomers are attracting much attention because of their potential role in disease pathogenesis and progression (Kayed et al., 2003; Lee et al., 2014; Outeiro et al., 2008). In fact, it is now widely accepted that aSyn is secreted and propagates

between neurons in a prion-like manner (Marques and Outeiro, 2012; Martin et al., 2012). Therefore, different aSyn species (monomer, oligomers, or fibrils) are predicted to gain access to the extracellular space and act postsynaptically, to impair neuronal communication and plasticity. Consistent with this hypothesis, we have previously shown that extracellular aSyn oligomers impair LTP, via NMDAR activation, before the occurrence of any neuronal death or changes in membrane conductance (Diógenes et al., 2012; Ferreira et al., 2015). Recent findings demonstrate that the cellular form of PrP (PrP^C) can act as a cell surface binding partner for β-sheet-rich protein aggregates, namely soluble oligomeric protein species (Beraldo et al., 2016; Caetano et al., 2011; Laurén et al., 2009; Resenberger et al., 2011). Moreover, PrP^C is involved in age-dependent behavioral abnormalities (Schmitz et al., 2014), memory impairment in animal models of neurodegeneration (Gimbel et al., 2010), and mediates Ca^{2+} influx via NMDAR (Khosravani et al., 2008). These observations suggest that PrP^C might act as a mediator of the synaptotoxic effects triggered by aSyn oligomers. We now establish a previously undocumented link between aSyn and PrP^C, whereby extracellular aSyn oligomers disturb Ca^{2+} homeostasis, impacting on synaptic plasticity. These toxic effects depend on PrP^C since LTP and calcium impairments are lost in *Prnp* null mice or by blocking PrP^C with an antibody. In addition, our data suggest that the amino acid region 93-109 of PrP^C is involved in mediating the toxic effects of aSyn. Whether the reported interaction also affects the spreading of aSyn in the brain (Urrea et al., 2017), still needs to be investigated.

It has been suggested that the putative receptor function of PrP^C relates to the modulation of phosphorylation cascades, in particular that governed by Fyn (Linden et al., 2008; Sorgato and Bertoli, 2009), a member of the Src Tyr-kinase (SFK) family, which highly expressed in neurons (Um and Strittmatter, 2013). Through this pathway, PrP^C is

thought to play a key role in the regulation of several cellular processes, ranging from embryogenesis to neuroprotective signaling (Wulf et al., 2017). In fact, under physiological conditions, PrP^C depresses Fyn activity and, consequently, attenuates Ca^{2+} influx via NMDAR (Khosravani et al., 2008). Our findings are in line with these results by demonstrating that the interaction of PrP^C with extracellular aSyn oligomers leads to SFK phosphorylation and, consequently, to NMDAR hyper-activation followed by a rise in postsynaptic Ca^{2+} levels. Moreover, either Fyn or PrP^C inhibition, completely prevent aSyn-mediated synaptic deficits. This toxic Fyn signaling cascade can be attributed to the relief of the PrP^C constitutive block of Fyn or, alternatively, to aberrant Fyn activation, as previously proposed for Aβ oligomers (Larson et al., 2012; Um et al., 2012). Our results support the latter, since P*rnp*$^{-/-}$ mice do not display LTP impairments even in the presence of aSyn oligomers, suggesting that no significant constitutive SFK inhibition occurred. Moreover, Fyn-mediated intracellular Ca^{2+} flux can occur via store-operated Ca^{2+} entry (SOCE) (De Mario et al., 2015) or via NMDAR (Khosravani et al., 2008). We demonstrate that the PrP^C-dependent effect on Ca^{2+} influx arises via NMDAR2B rather than SOCE, since NMDAR2B blockade rescued Ca^{2+} increase and both PrP^C and SFK blockade prevented NMDAR2B phosphorylation, induced by aSyn.

aSyn/PrP^C and SFK cannot interact directly, given that PrP^C is extracellularly anchored to the plasma membrane, whereas SFK is cytosolic. Our data identifies mGluR5 as the protein linking PrP^C and SFK on opposite sides of the plasma membrane. Previous studies demonstrated that mGluR5 mediates Fyn activation promoted by PrP^C (Um and Strittmatter, 2013). Consistently, when we blocked mGluR5 activation we prevented aSyn-induced NMDAR2B phosphorylation and synaptic impairment. Conversely, we bypassed the PrP^C blockade of the aSyn

effects by activating mGluR5 directly, suggesting mGluR5 acts downstream of aSyn/PrP^C and upstream of Fyn/NMDAR2B activation.

Interestingly, the identification of the mGluR5-Fyn-NMDAR2B pathway as a mediator of the aSyn-PrP^C signaling uncovers new targets for therapeutic intervention. However, blocking directly Fyn kinase, PrP^C or NMDAR interferes with basal neuronal function and impairs memory, even in WT mice, as these proteins are crucial components of the postsynaptic density (Khosravani et al., 2008). Accordingly, *Prnp*$^{-/-}$ exhibit deficits in hippocampal-dependent spatial learning, alterations in hippocampal physiology (Criado et al., 2005), synaptic alterations (Collinge et al., 1994; Curtis et al., 2003), social recognition memory deficits, impaired motor coordination, and activity (Katamine et al., 1998; Schmitz et al., 2014). Thus, this deleterious phenotype of the *Prnp*$^{-/-}$ mice may occlude any attempt to rescue *in vivo* aSyn-induced toxicity in a *Prnp* null background. Likewise, a failure to rescue this particular aSyn phenotype by using a Fyn kinase inhibitor, PrP^C antibody, or NMDAR antagonists *in vivo* would prove inconclusive, since their constitutive activity is essential for synaptic function. Another alternative for rescuing memory and synaptic impairments *in vivo*, would be to interfere with mGluR5. mGluR5 are key players in cognitive and synaptic plasticity processes, and their direct antagonism impairs LTP and memory *in vivo* (Um et al., 2013). Yet, their functional interaction with adenosine $A_{2A}R$, which regulate mGluR5-mediated effects via NMDAR2B phosphorylation (Sarantis et al., 2015; Tebano et al., 2005), provides a suitable alternative for regulating aberrant mGluR5 signaling without disrupting its constitutive activity. A similar approach was used to determine the involvement of PrP^C in synaptic impairment driven by Aβ oligomers (Um et al., 2013).

We now demonstrate the ability of an $A_{2A}R$ antagonist (KW-6002) to rescue synaptic and cognitive deficits in aSyn-transgenic mice,

providing the crucial evidence that the toxic effects of aSyn are indeed modulated by downstream effectors of PrP^C (mGluR5/Fyn), as we described *in vitro*. KW-6002, also known as istradefyline, is approved in Japan for the adjunctive treatment of motor deficits in PD (Dungo and Deeks, 2013) and was shown to be particularly suited to target the CNS, based on its bioavailability, half-life, and brain penetration in animal studies (Yang et al., 2007).

Previous evidence that blockade of $A_{2A}R$ effectively inhibits NMDAR2B phosphorylation evoked by mGluR5 (Tebano et al., 2005) and prevents LTP impairment and NMDAR overactivation mediated by aSyn *in vitro,* as shown in chapter II of this thesis (Ferreira et al., 2015) further supports this crosstalk**.** More importantly, deletion of $A_{2A}R$ is protective against neuronal degeneration induced by a mutant human α-synuclein (hm(2)-αSYN) transgene, although the underlying mechanisms were unknown at the time (Kachroo and Schwarzschild, 2012).

Our evidence that KW-6002 treatment normalizes NMDAR2B and PrP^C levels in Thy1-aSyn mice, preventing Fyn phosphorylation while rescuing memory and LTP, is very relevant to support our hypothesis and the involvement of this pathway *in vivo* **(Fig. 3.7**). Also, the fact that aSyn was found to be increased in cerebrospinal fluid of prion disease patients reinforces the pathophysiological relevance of our findings (Kasai et al., 2014).

Importantly, at the time of treatment, the aSyn transgenic mice used already displayed detectable cognitive deficits (Magen et al., 2012), suggesting that memory impairments elicited by aSyn overexpression are reversible and do not result from cell death. Indeed, aSyn mice only present neuronal loss later in life (Chesselet et al., 2012). In accordance, we show that aSyn oligomers activate SFK/NMDAR as early as 5 min after exposure, while neuronal death occurs only after 24 h of exposure, as demonstrated in chapter II results (Ferreira et al., 2015). These data support

the hypothesis that a receptor-mediated mechanism, independent of pore formation and membrane leakage (Di Scala et al., 2016b), is sufficient to trigger early synaptic damage induced by extracellular aSyn. Furthermore, we identified novel key players in the signaling cascade triggered by aSyn, suggesting PrP^C signaling may play a role in early stages of PD and DLB.

In total, our findings provide novel options for therapeutic intervention aimed at neutralizing the downstream effects taking place at synapses, rather than relying solely on the disease-causing agent. Thus, this strategy may prove more effective at preventing or delaying the onset of synucleinopathy-associated memory deficits.

Materials and Methods

Animals

Animal procedures were performed in accordance with the European Community guidelines (Directive 2010/63/EU), Portuguese law on animal care (DL 113/2013), and approved by the Instituto de Medicina Molecular Internal Committee and the Portuguese Animal Ethics Committee (Direcção Geral de Veterinária). Environmental conditions were kept constant: food and water ad libitum, 21 ± 0.5°C, 60 ± 10% relative humidity, 12 h light/dark cycles, 2 to 3 rats per cage or 3 to 4 mice per cage. Only male animals were used in all experiments. Mice were sacrificed by cervical dislocation and rats sacrificed by decapitation after anesthesia under halothane atmosphere. Male Sprague Dawley rat (SD; Harlan, Barcelona, Spain) with 8-12 weeks old were used for electrophysiological experiments. Tg mice overexpressing human aSyn under the Thy-1 promoter were generated on a mixed C57BL/6-DBA/2 background as described previously (Rockenstein et al., 2002). Animals were maintained on this background by breeding mutant females with type (WT) with C57BL/6-DBA/2 males. Offspring were genotyped with polymerase chain reaction (PCR) amplification analysis of tail DNA (40 cycles, 60°C annealing temperature). The sequences of primers used were: Thy-1-F: 5'-CTG GAA GAT ATG CCT GTG GA-3', Thy-1-R: 5'-GAG GAA GGA CCT CGA GGA AT-3' (Invitrogen). Male Tg Thy1-aSyn mice and their WT littermates with matched age (6-8 month old) were used for behavioral experiments. Prion protei knockout mice (designated *Prnp*$^{-/-}$, or *Zurich I*) homozygous for the disrupted *Prnp* gene, were produced on a mixed C57BL/6J x 129/Sv background as previously described (Büeler et al., 1992). Male *Prnp*$^{-/-}$ mice and their WT littermates with matched ages

(6-8 month old) were used for electrophysiological and molecular biology experiments.

Oral administration of the drug

KW-6002 (istradefylline; Tocris Bioscience), a selective adenosine $A_{2A}R$ antagonist (Yang et al., 2007), was orally administered, diluted in the drinking water, being continuously available, as before (Batalha et al., 2013; Coelho et al., 2014). The weight of the animals and the volume intake were assessed twice a week and the concentration of the solution was adjusted so that the drug intake was maintained at 3 mg/kg per day. Animals were divided into four groups: WT mice drinking vehicle (0.025% methylcellulose) or drinking KW-6002 (3 mg/kg per day, 0.025% methylcellulose), and aSyn Tg mice drinking vehicle or KW-6002 at the same concentration (**Fig. 3.5a**). The treatment started at 5 months old, one month until behavior assessment started. The KW-6002 administration was kept until sacrifice.

Behavioral assessments

Mice were first handled for 5 days prior to behavioral tests. Mazes were cleaned with a 70% ethanol solution between each animal. Animals were randomized, and the experimenter blinded to genotype for the duration of behavioral testing. All behavioral tests were performed during the light phase between 8 a.m. and 6 p.m. in a sound attenuated room.

Y-maze behavioral assessment. Short-term reference memory was assessed in a spontaneous novelty-based spatial preference Y-maze test. The Y-maze was performed in a two-trial recognition test in a Y-shaped maze with 3 arms (each with 15 cm length x 5 cm wide x 12 cm height), angled at 120º and with opaque walls. Different cues were placed on the surrounding walls. Allocation of arms was counterbalanced within each

group. During the first trial (learning trial), mice were placed at the end of the "start" arm and were allowed to explore the maze for 10 min with only two arms opened ("start" and "other" arm). Access to the third arm of the maze ("novel" arm) was blocked by an opaque door. The mouse was then removed from the maze and returned to its home cage. After 1 h, the animal was placed again in the "start" arm of the maze, the door of the "novel" arm was removed, and the mouse was allowed to explore the maze for 5 min (test trial). Mice tracings were continuously monitored by an automated tracking system (Smart 2.5, PanLab, Barcelona). Preference for the novel arm is considered a measure of short-term reference memory. To exclude the possible confounding effect of alterations of locomotor activity, we used the frequency of entrance into the arms (number of transitions) as an indirect indicator of the general locomotor activity. The animal's behavior was performed by an observer blind to the treatment conditions and genotype.

Morris water maze (MWM). Spatial memory ability was evaluated in the MWM test (Morris et al., 1982). The test was performed over the course of six consecutive days and consisted of a five-day acquisition phase and a one-day probe test. The test was performed in a circular pool, with 100 cm in diameter, filled with water opacified with non-toxic white paint (Luxens) and kept at 24ºC. A round 8-cm in diameter platform was hidden 1 cm beneath the surface of the water at a fixed position. Four positions around the edge of the tank were used, dividing the tank into four quadrants: target quadrant (T, quadrant here the platform was hidden), left quadrant (L, quadrant on the left of the target quadrant), right quadrant (R, quadrant of the right of the target quadrant) and opposite quadrant (O, quadrant on the opposite side of the target quadrant). During the acquisition phase, each mouse was given four swimming trials per day (30-min intertrial interval). A trial consisted of placing the mouse into the water facing the outer edge of the pool and allowing the mouse to explore

and reach for the hidden platform. If the animal reached the platform before 60 secs, it was allowed to remain there for 10 secs, if the animal failed to find the target before 60 secs, it was manually guided to the platform, where it was allowed to remain for 20 secs. After the end of each trial, mice were removed from the pool and placed back into their home cages beneath heat lamps in order to prevent temperature loss. On the probe test, the platform was removed, and animals were allowed to swim freely for 60 secs while recording the percentage of time spent in each quadrant. The latency to find the platform during the acquisition phase and the percentage of time in the platform quadrant in the probe test were recorded and analyzed using the Smart 2.5 tracking system (PanLab, Barcelona) and used to evaluate hippocampal-dependent memory. Swimming speed, measure of possible motor defects that could interfere with the ability to perform the task, was also registered.

Histological procedures

Mice were transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS (0.1 mol/L PBS, pH 7.4) under deep pentobarbital anesthesia. Brains were removed, post-fixed for 24 h in 4% paraformaldehyde, embedded in paraffin and cut into coronal sections of 1.5 μm. For immunohistochemistry, slides were deparaffinized, rehydrated and antigen retrieval was performed by heating at 70ºC for 1 hour in 0.01M Citrate Buffer pH 6. Slices were incubated with primary antibodies specific for aSyn (1:200, mouse monoclonal IgG1, BD Biosciences, 610787) and SNAP25 (1:5000, rabbit polyclonal IgG, Sigma-Aldrich, S9684) or PrP^C (C-20, 1:50; goat polyclonal IgG, Santa Cruz Technology, #sc-7693) overnight at room temperature and washed with PBS before being incubated overnight at room temperature with secondary antibodies (Alexa Fluor 568 donkey anti-mouse and Alexa Fluor 488

donkey anti-rabbit or anti-goat, 1:400, Invitrogen). After washing, slices were incubated with Hoechst (Hoechst 33342, Thermo Scientific; 12 µg/ml final concentration), washed once and mounted in Dako Fluorescent Mounting Medium (Dako). Z-stack images at 63x magnification were acquired with a Zeiss LSM 880 Confocal Microscope and compositional images of hippocampal formation were produced by tile stitching of images at 10x magnification acquired using Zeiss Cell Observer Widefield Fluorescence Microscope. For tyrosine hydroxylase (TH) immunohistochemistry, coronal sections of 4 µm were used. Slices were then deparaffinized, rehydrated and antigen retrieval was performed by heating at 70ºC for 1 hour in 0.01M Citrate Buffer pH 6. Slices were incubated with primary antibody specific for TH (1:750, rabbit polyclonal IgG, abcam, ab112). Quantification of the TH staining intensity was performed using the NDP view Software (Hamamatsu Photonics, France).

Electrophysiological fEPSPs recordings

The experiments were performed in acute transverse hippocampal slices from male SD rats (8–12 weeks old), in *Prnp*$^{-/-}$ mice, and in Thy1-aSyn (aSyn Tg) mice and their respective WT littermates. The experimenter was blind to genotype and/or treatment. After decapitation, the brain was rapidly removed, and the hippocampi were dissected free in ice-cold artificial CSF, known also as Krebs solution, which is composed of (mM): NaCl 124; KCl 3; NaH_2PO_4 1.25; $NaHCO_3$ 26; $MgSO_4$ 1; $CaCl_2$ 2; and D-glucose 10, previously gassed with 95% O_2 and 5% CO_2, pH 7.4. Slices (400 µm thick) were obtained with a McEwan tissue chopper and were pre-incubated with or without extracellular aSyn oligomers (500 nM) for 90 min at RT in gassed artificial CSF. Treatment with the different drugs started 20 min prior to aSyn oligomers pre-incubation and was kept throughout the 90 min of aSyn pre-incubation (**Fig. 3.1b, 3.2a, 3.4a, c**).

The range of concentration and time-course chosen for aSyn incubation was based on previous data in which we tested and optimized for obtaining the max effect without compromising the slice viability crucial for the electrophysiology recordings (Diógenes et al., 2012). In a set of preliminary experiments, we tested 10–50 nM aSyn in fEPSPs but did not detect significant changes. Following this incubation period, slices were then washed, placed in the recording chamber and superfused with artificial CSF (3 mL/min) at 32°C. fEPSPs were recorded in the *stratum radiatum* of the CA1 area (**Fig. 3.1a**), as previously described (Diógenes et al., 2012). An input-output (I/O) protocol was performed to determine the synaptic response parameters for each slice. The Schaffer collaterals were stimulated (stimulus rate of 1 pulse per 30 sec) at a range of stimulus intensities. The stimulus strength was increased until the maximum population spike amplitude was reached. The I/O curve was plotted as the relationship of fEPSP slope versus stimulus intensity, which provides a measure of synaptic efficiency. The max slope values were obtained by extrapolation upon nonlinear fitting of the I/O curve and an *F*-test was used to determine differences between the parameters. Short-term synaptic plasticity at the dendritic synapses was assessed by measuring paired-pulse facilitation (PPF) using a standard paired-pulse stimulation protocol applied to the Schaffer collaterals. Paired-pulse interval of 200 ms was used at a test stimulus intensity that elicited a fEPSP equal to 50% of the maximal fEPSP amplitude, as determined from I/O protocols. Three paired-pulse responses were averaged in each slice. Long-term plasticity was evaluated by a long-term potentiation (LTP) and a long-term depression (LTD) protocol. LTP was induced by a theta-burst stimulation protocol (TBS, 10 trains with 4 pulses each at 100 Hz, separated by 200 ms) applied to the Schaffer collaterals at the test stimulus intensity (50% half-maximal fEPSP). Long-term depression (LTD) was induced using a low frequency stimulation protocol (LFS, 3 trains with 10 min interval of

2 Hz, 1200 pulses) as previously described (Laurent et al., 2014). The specificity of these effects was previously validated by testing the same concentration of insulin oligomers, which caused no significant changes in LTP magnitude (Diógenes et al., 2012). Stimulation, data acquisition and analysis were performed using the electrophysiology software program WinLTP program or pClamp (Molecular Devices).

Primary neuronal cultures

Hippocampal neurons were cultured from 18 days Sprague Dawley rat (Harlan, Barcelona, Spain) and *Prnp*$^{-/-}$ mice embryos as previously described (Pedersen et al., 2002; Valadas et al., 2012). Briefly, embryos were collected in Hank's Balanced Salt Solution (HBSS, Corning) and rapidly decapitated. Meninges were removed, and whole cortices (hippocampi and attached cortex) were dissociated and incubated for 15 minutes in HBSS with 0.025% trypsin. Cells were washed once with HBSS with 30% Fetal Bovine Serum (FBS), centrifuged three times, re-suspended in Neurobasal Medium (Gibco – Life Technologies) supplemented with 2% B-27 supplement, 25 μM Glutamate, 0.5 mM glutamine, and 2 U/ml Penicillin/Streptomycin, gently dissociated and filtered through a 70μm strainer (VWR). Cells were plated on poly-D-lysine-coated plates and grown for 10 days at 37°C in a 5% CO_2-humidified atmosphere in the previously described supplemented Neurobasal medium, in the absence of any positive selection for neurons. Medium was not replaced, and cultures were treated with aSyn species (500 nM) and drugs at day 12.

Ca^{2+} imaging

Primary neuronal cultures from WT and *Prnp*$^{-/-}$ mice were plated at a density of 20 x 10^3 cells per well in glass bottom microwell chambers

previously coated with poly-D-lysine. At the 12-16 DIV neurons were loaded with Fura-2 AM (5 µM, in external physiological solution with the following composition in mM: NaCl 125, KCl 3, NaH_2PO_4 1.25, $CaCl_2$ 2, $MgSO_4$ 2, D-(+)-glucose 10 and HEPES 10; pH 7.4 adjusted with NaOH) and incubated at 37ºC for 1 h. Cells were then placed on a heated chamber installed in an inverted microscope with epifluorescent optics and equipped with a high speed multiple excitation fluorimetric system (Lambda DG4, with a 175W Xenon arc lamp). Data was recorded by a CDD camera. Fura-2 AM leaded neurons were sequentially excited both at 340 nm and 380 nm, for 250 ms at each wavelength, and the emission fluorescence was recorded at 510 nm. Experiments were performed on cells with a baseline fluorescence ratio around 0.5, which corresponds approximately to a $[Ca^{2+}]i$ of about 100 nM, considered the normal $[Ca^{2+}]i$ (Barhoumi et al., 2010; Knot, 2005). Cells with a baseline fluorescence ratio above 1 were discarded from the experiment. Experiments were performed at 37ºC in a 5% CO_2-humidified atmosphere. Drugs, anti-PrP^C antibodies and aSyn species were applied directly to the cells medium. All cells were challenged with ionomycin (an effective Ca^{2+} ionophore) at the end of the experiment and only those that responded were included, confirming neuronal viability. Image data were recorded and analyzed using the MetaFluor software (Universal Imaging, West Chester, PA, USA).

Immunocytochemistry

Primary neuronal cultures with 12 DIV were fixed for 10 minutes with 4% Paraformaldehyde diluted in Phosphate Buffer Saline (PBS). After washing with PBS, cells were permeabilized for 10 minutes with 0.05% Triton-X in PBS, blocked for 30 minutes with 10% FBS in PBS and incubated overnight at 4°C with the mature neuronal marker, rabbit anti-

MAP-2 (microtubule-associated protein 2; abcam ab32454; 1:200 dilution), and the astrocytic and the immature neuronal marker, mouse anti-GFAP antibody (glial fibrillary acidic protein; Millipore MAB360; 1:250 dilution) or, with the primary antibodies specific for aSyn (1:50, rabbit polyclonal IgG, Cell Signaling Technology, #2628S) and PrP^C (1:50; Santa Cruz Technology, #sc-7693), diluted in PBS with 0.05% Tween-20 (PBS-T) and 4% FBS. After washing with PBS-T, cells were incubated for 1 hour with Alexa Fluor 488 donkey anti-rabbit and Alexa Fluor 568 donkey anti-mouse or anti-goat antibodies (Invitrogen) diluted 1:400 in PBS-T with 4% FBS. In order to label cell nucleus, after washing with PBS-T, coverslips were incubated for 5 minutes with Hoechst (Hoechst 33342, Thermo Scientific; 12μg/ml final concentration) and washed for 30 minutes with PBS -T. After a final washing step with PBS, coverslips were mounted with Dako Fluorescent Mounting Medium (Dako) and let to dry for 24h at room temperature, protected from light exposure. Cells were observed with a Zeiss Cell Observer Widefield Fluorescence Microscope and a Zeiss LSM 880 Confocal Microscope. For culture characterization purposes, fifteen arbitrary photographs were acquired at 20x magnification and different cell subsets were counted and analyzed using Image-J software (NIH, Bethesda, MD, USA). Approximately, 50% of cells were mature neurons (MAP-2 positive cells) and 30% were GFAP positive cells. The remaining cells were microglia, as confirmed by nuclei identification based on their morphological characteristics (Garman, 2011).

Purification and oligomerization of recombinant aSyn

aSyn was prepared as previously (Diógenes et al., 2012; Vicente Miranda et al., 2013). Monomeric aSyn was readily used or stored at −80°C until further use. Oligomerization was induced by continuous shaking of

monomeric aSyn (140 µM) for 6 days at 37°C in a thermomixer (Eppendorf) at 900 rpm. Samples were ultracentrifuged to obtain fibrillary aSyn. The supernatant containing monomeric and oligomeric aSyn was centrifuged in Amicon filter unit with Ultracel membrane NMWL of 30 kDa (Millipore). The fibrillary aSyn (>180 kDa) and the retained fraction containing aSyn oligomers (> 30 kDa) was readily used or stored at −80°C in small aliquots to avoid freeze/thaw cycles until further use. The concentration of aSyn was determined using its molar extinction coefficient at 280 nm (i.e., ε280 = 5960 L/mol/cm). The composition of different aSyn species, monomers, oligomers and fibrils was evaluated by SDS–PAGE (**Supplementary Fig. 3.1a**). Five micrograms of each aSyn sample was separated by SDS–PAGE using a Tetra Cell (Bio-Rad) in a precast 4–15% polyacrylamide gel (Bio-Rad), using standard procedures. To ensure consistency and stability of the effects of the oligomer preparation, each batch was pre-screened for toxicity using LTP as readout prior to any further testing.

Co-immunoprecipitation (Co-IP)

Briefly, WT rat hippocampal slices were homogenized in IP buffer (NP40 1%, SDS 0.1%, Tris–HCl 50 mM, NaCl 150 mM, sodium deoxycholate 0,5%, EDTA 1 mM, protease inhibitors - Complete, EDTA-free Protease Inhibitor cocktail tablets; Roche) (pre-IP lysates, see in Supplementary Material). Protein extracts were incubated with protein G PLUS-Agarose (Santa Cruz, Biotechnology) for 1 h at 4°C to eliminate nonspecific binding. After incubation, the precleared supernatants containing 1 mg of protein were incubated with anti-PSD-95 antibody (1:50; Cell Signaling Technology, #D27E11), anti-PrP C-20 (1:50; Santa Cruz Technology, #sc-7693), anti-aSyn (1:50; Cell Signaling Technology, #4179), anti-α-tubulin (1:50, Abcam, #ab52866), or IgG (for negative control; Santa Cruz

Biotechnology) overnight at 4°C under rotation. The day after, lysates were incubated with protein G PLUS-Agarose for 3 h with rotation at 4°C. Beads were washed 3 times with IP buffer and resuspended in 1.5× sample buffer pH (Tris 70 mM pH 6.8, glycerol 6%, sodium dodecyl sulfate 2%, dithiothreitol 120 mM, and bromophenol blue 0.0024%). Pre-IP lysates and bound proteins eluted from the immune complexes were denatured by heating to 95°C for 5 min and used for western blot analysis.

Western Blotting

Neuronal cells were washed with cold PBS and then mechanically scrapped in radioimmunoprecipitation assay buffer pH 8.0 (RIPA buffer: NaCl 150 mM, Tris-base 50 mM, EDTA 1 mM, Nonidet P40 1%, sodium dodecyl sulfate 0.1%, proteases inhibitors - Complete, EDTA-free Protease Inhibitor cocktail tablets; Roche). Hippocampus from Thy1-aSyn (aSyn Tg) and WT mice were homogenized in the same buffer by sonication. After protein quantification using BioRad DC Protein Assay kit, lysates were denatured with 5x sample buffer pH 6.8, as above and heated at 95°C for 5 min and further processed as before (Valadas et al., 2012). Samples and the prestained molecular weight marker (BIO-RAD) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE; 15% gel) under reducing conditions and electro-transferred to polyvinylidene difluoride membranes (0.45 µm, GE Healthcare Life Sciences) using standard procedures. Thereafter, nonspecific binding was blocked with 3% bovine serum albumin (fatty acid free) in Tris-buffered saline (pH 7.6) containing 0.1% Tween 20 (TBS-T) for 1 h at RT. Membranes were then incubated overnight at 4°C with the corresponding primary antibody, namely mouse anti-aSyn (1:1000; BD Biosciences, #610787), rabbit anti-aSyn (1:1000; Cell Signaling Technology, #4179), rabbit anti-α-tubulin (1:5000; Abcam,

#ab52866), mouse anti-GAPDH (6C5; 1:1000; ThermoFisher Scientific, #AM4300), rabbit anti-PSD-95 (1:1000; Cell Signaling Technology, #2507), rabbit anti-NMDA receptor subunit 2B (D15B3; 1:1000; Cell Signaling Technology, #4212), rabbit anti-phospho-NMDA receptor subunit 2B (Tyr1472; 1:1000; Cell Signaling Technology, #4208), mouse anti-NMDA receptor subunit 1 (1:500; BD Biosciences, #556308), rabbit anti-Fyn kinase (H-80; 1:1000; Santa Cruz Biotechnology, #sc-28791), rabbit anti-phospho-Src (Tyr416; 1:100; Cell Signaling, #2101), mouse anti-PrP 6D11 (6D11; 1:1000; BioLegend, #SIG-39810), goat anti-PrP C-20 (C-20; 1:500; Santa Cruz Biotechnology, #sc-7693) and/or mouse anti-tyrosine hydroxylase (TH; 1:200; abcam, #ab112) diluted in blocking solution. After 3 washing periods of 10 min with TBS-T, membranes were incubated with horseradish peroxidase (HRP) - conjugated anti-mouse, anti-rabbit or anti-goat secondary antibodies (1:10 000; Santa Cruz Biotechnology) (in 5% nonfat dry milk) for 1 h at RT. After 40 min of washing with TBS-T, chemiluminescent detection was performed with ECL western blotting detection reagent (GE Healthcare Life Sciences) using X-Ray films (Fujifilm). Densitometric quantification was determined using Image-J software and normalized to the corresponding control band density.

Drugs and PrP^C antibodies

The mGluR5-selective agonist, (S)-3,5-Dihydroxyphenylglycine (DHPG), and the ionophore Ionomycin were purchased from Sigma-Aldrich. The $A_{2A}R$-selective antagonists, (E)-8-(2-(3,4-dimethoxyphenyl)-vinyl)-1,3-diethyl-7-methyl-3,7-dihydropurine-2,6-dione (KW-6002, istradefylline), and 5-Amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5c]pyrimidine (SCH-58261) plus the mGluR5-selective antagonist, 2-Methyl-6-(phenylethynyl)pyridine (MPEP), were purchased

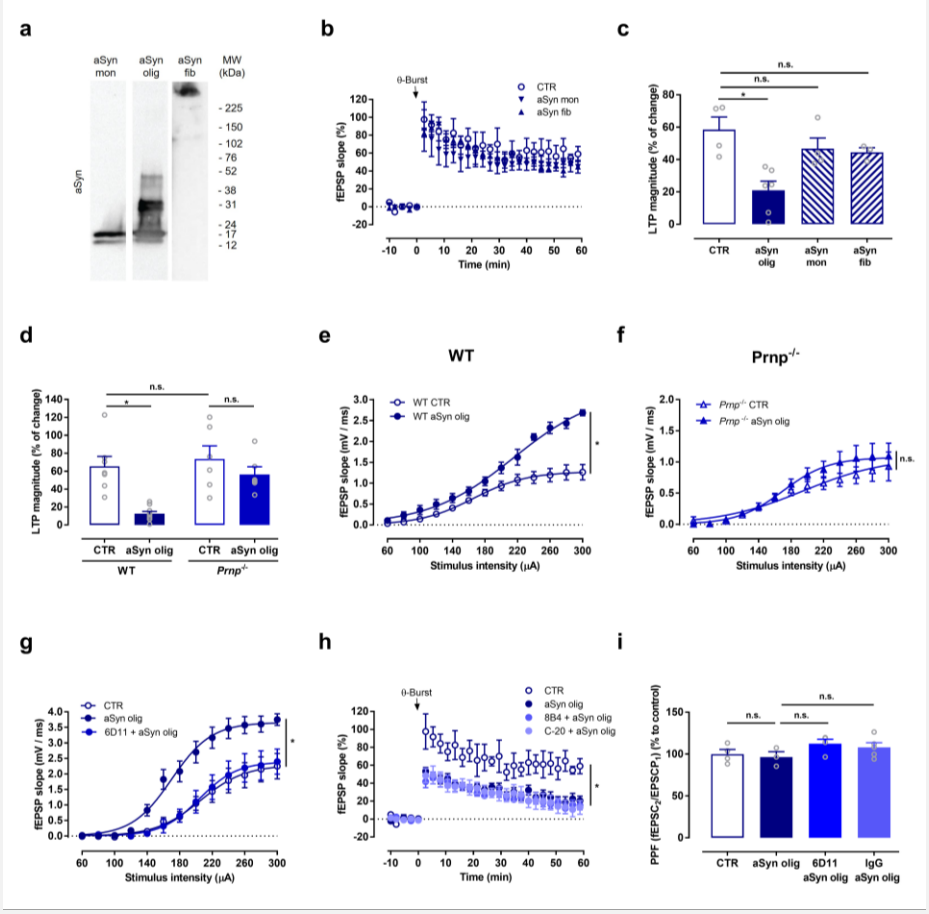
from Tocris Bioscience. The Src-family inhibitor 1-(1,1-Dimethylethyl)-3-(1-naphthalenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (1-Naphthyl PP1), the selective NMDAR antagonist, DL-2-Amino-5-phosphonopentanoic acid (APV), and the selective NMDAR subunit NR2B, Ifenprodil, were purchased from Abcam.

The following anti-PrP antibodies were used: 6D11 (mouse monoclonal; epitope targeting between amino acids 93 and 109; BioLegend, #SIG-39810), C-20 (goat polyclonal; epitope targeting the C terminus; Santa Cruz Biotechnology, #sc-7693), and 8B4 (mouse monoclonal; Santa Cruz Biotechnology, epitope targeting the N-terminus, #sc-47729).

Statistical analysis

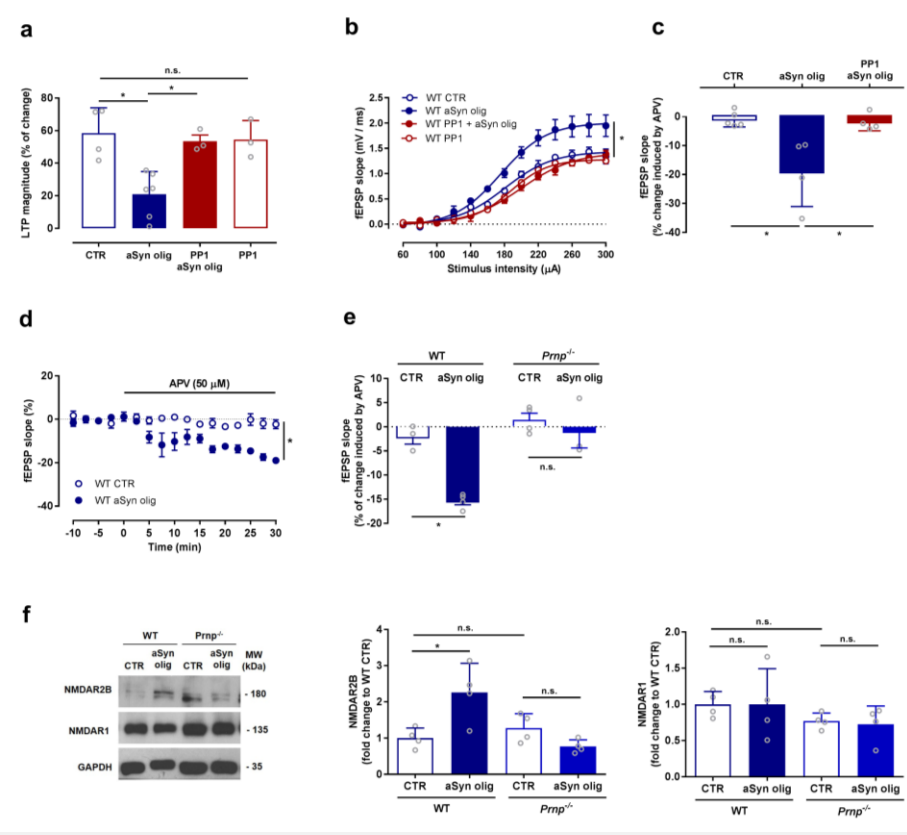
All statistical analyses were performed with GraphPad Prism software. Values are presented as dot blots with individual values plus bar, with mean ± s.e.m. in figure legends. Statistical analyses were designed using the assumption of normal distribution and similar variance among groups, as previously tested. Statistical comparisons included two-sided unpaired *t* test, one or two-way ANOVA followed by a Bonferroni's, Dunnett's or Tukey's multiple comparison post hoc tests as specified in the figure legends. *P*-values of < 0.05 were considered statistically significant. The sample size was determined based on preliminary results or similar experiments carried-out in the past. Power Analysis was performed using G-power in order to estimate the number of animals required, for a signal-to-noise ratio of 1.4 and 80% to 90% power assuming a 5% significance level.

Supplementary information



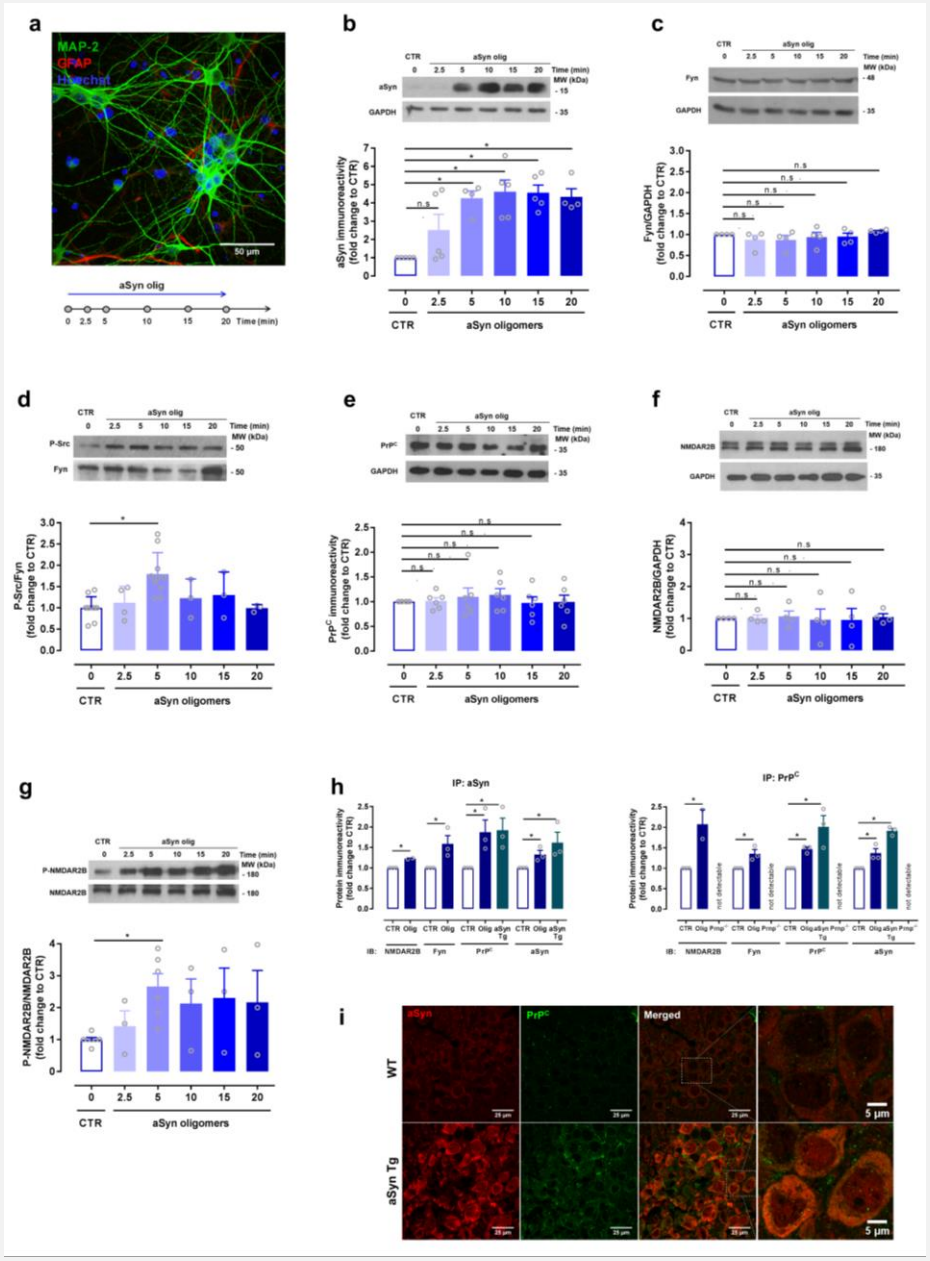
Supplementary Figure 3.1. Characterization of the aSyn species and biological effects. (**a**) SDS–PAGE separation of the different aSyn species. Monomers (aSyn mon) migrate with monomeric molecular weight (15 kDa) whereas aSyn oligomers (aSyn olig), and fibrils (aSyn fib) display SDS-resistant high-molecular weight species. (**b**) Changes in fEPSP slope induced by theta-burst stimulation recorded from WT rat hippocampal slices pre-incubated with extracellular aSyn monomers (90 min, 500 nM, $n = 4$), fibrils (90 min, 500 nM, $n = 3$) or in control conditions (CTR, $n = 4$). (**c**) Plot of the LTP magnitude represented in **b** (change in fEPSP slope at 50–60 min after theta-burst stimulation, compared to baseline) (means ± s.e.m., $P < 0.01$, one-way ANOVA followed by a Bonferroni's Multiple Comparison Test). (**d**) Plot

of the LTP magnitude obtained from WT and *Prnp*$^{-/-}$ hippocampal slices pre-incubated with extracellular aSyn oligomers (n = 10, 6) or in control conditions (n = 7, 6) (means ± s.e.m., $P < 0.001$, one-way ANOVA followed by a Bonferroni's Multiple Comparison Test). (**e**) Input/Output (I/O) curves corresponding to fEPSP slope evoked by various stimulation intensities (10 – 120 µA) from WT hippocampal slices pre-incubated with or without aSyn oligomers (n = 5, 7; means ± s.e.m., $P < 0.001$, F-test). (**f**) I/O curves from *Prnp*$^{-/-}$ hippocampal slices pre-incubated with (n = 4) or without (n = 4) aSyn oligomers obtained by the same method as in **e** (means ± s.e.m., $P > 0.05$, F-test). (**g**) I/O curves obtained by the same method as in **e**, from hippocampal slices CTR (n = 6), pre-incubated with aSyn oligomers alone (n = 7) or in the presence of the anti-PrP 6D11 antibody (6D11 + aSyn olig, 110 min, 100 nM, n = 4) (means ± s.e.m., $P < 0.01$, F-test). (**h**) Changes in fEPSP slope, obtained by the same methods as in **b**, from WT hippocampal slices in control conditions (n = 4) and in the presence of aSyn oligomers alone (n = 6) or together with the anti-PrP antibodies, 8B4 (8B4 + aSyn, 110 min, 10 µg, n = 4) or C-20 (C-20 + aSyn olig, 110 min, 10 µg, n = 4) (means ± s.e.m., $P < 0.001$, one-way ANOVA followed by a Bonferroni's Multiple Comparison Test). (**i**) Paired Pulse Facilitation (PPF) plotted against 200 ms interpulse intervals in WT slices submitted to the same conditions as in **g** (n = 3-5; means ± s.e.m., $P > 0.05$, one-way ANOVA followed by a Bonferroni's Multiple Comparison Test).



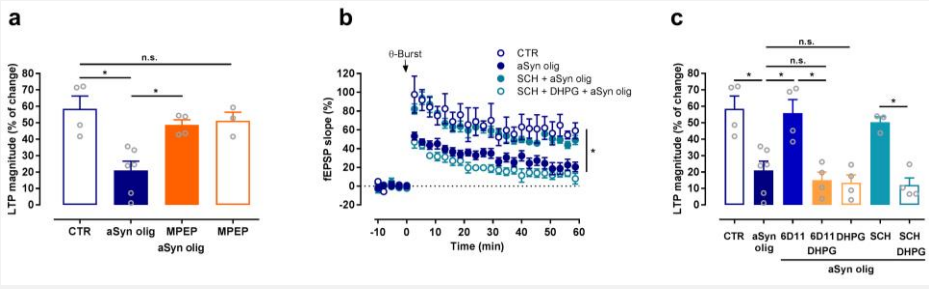
Supplementary Figure 3.2. Src pharmacological blockade prevents aSyn oligomer-induced synaptic impairment. (**a**) Plot of the LTP magnitude (change in fEPSP slope at 50–60 min after theta-burst stimulation, compared to baseline) from control WT hippocampal slices (CTR, *n* = 4), slices pre-incubated with the Src antagonist 1-naphthyl-PP1 (PP1, 110 min, 30 µM, *n* = 3) and slices pre-incubated with extracellular aSyn oligomers alone (aSyn olig, 90 min, 500 nM, *n* = 6) or in the presence of PP1 (PP1 + aSyn olig, *n* = 3) (means ± s.e.m., $P < 0.01$, one-way ANOVA followed by a Bonferroni's Multiple Comparison Test). (**b**) I/O curves corresponding to fEPSP slope evoked by various stimulation intensities (10 – 120 µA) from control WT hippocampal slices (CTR, *n* = 5) and slices pre-incubated with extracellular aSyn oligomers alone (*n* = 5) or in the presence of PP1 (*n* = 4) (means ± s.e.m., $P < 0.001$, *F*-test). (**c**) Quantification of the effects of the NMDAR antagonist APV (50 µM, 30 min) perfusion on basal fEPSP slope from control WT hippocampal slices (*n* = 5), and slices pre-incubated with extracellular aSyn

oligomers alone (n = 4) or in the presence of PP1 (PP1 + aSyn olig, n = 4) (change in slope between baseline and the last 10 min of APV application) (means ± s.e.m., P < 0.01, one-way ANOVA followed by a Bonferroni's Multiple Comparison Test). (**d**) Effect of APV (50 μM, 30 min) perfusion on basal fEPSP slope in WT hippocampal slices in control conditions (n = 3) or in the presence of aSyn oligomers (n = 4). (**e**) Quantification of the APV perfusion effects on basal fEPSP slope (change in slope between baseline and the last 10 min of APV application) from WT and *Prnp*$^{-/-}$ hippocampal control slices (CTR) or slices pre-incubated with aSyn oligomers (n = 3-4; means ± s.e.m., P < 0.001). (**f**) Representative immunoblot and quantification of NMDAR subunit 2B (NMDAR2B) and NMDAR subunit 1 (NMDAR1) levels in hippocampal slices from WT and *Prnp*$^{-/-}$ mice in the same conditions as in **d** (n = 4; means ± s.e.m., P < 0.001, one-way ANOVA followed by a Bonferroni's Multiple Comparison Test). GAPDH was used as a loading control.

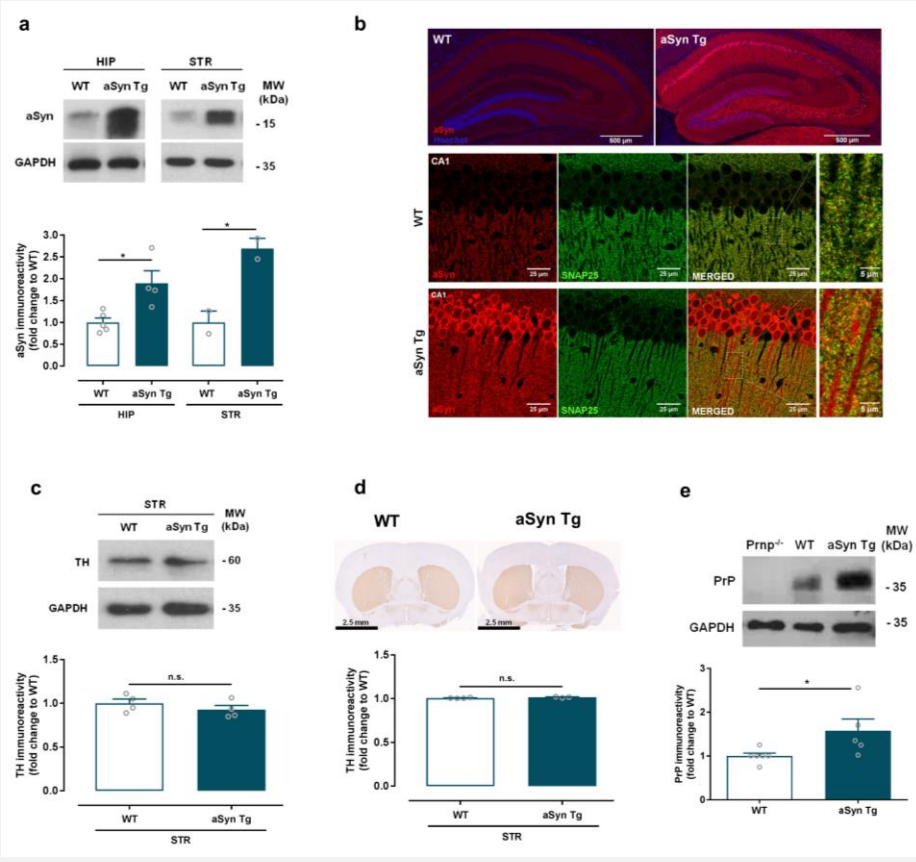


Supplementary Figure 3.3. Extracellular aSyn oligomers induce phosphorylation of SFK kinases and NR2B subunit of NMDAR. (**a**) Representative image of primary cultures from WT animals at 12 DIV. Mature neurons are labeled with green fluorescence with MAP-2 antibody, astrocytes, in red, are probed with anti-GFAP antibody, and cell nucleus are labeled with Hoechst 33342, in blue fluorescence. Z-

stack images were acquired using a confocal microscope at 40x magnification and converted into maximum intensity projections. At the bottom a schematic representation of the aSyn incubation protocol used. (**b, c**) Representative immunoblots and quantification of the aSyn and Fyn levels in neuronal cultures incubated with extracellular aSyn oligomers over time (n = 4-5, n = 4; $P < 0.01$, $P > 0.05$, one-way ANOVA followed by a Dunnett's Multiple Comparison Test). GAPDH was used as a loading control. (**d**) Representative immunoblots and quantification of the SFK kinases phosphorylation levels, normalized to Fyn immunoreactivity, in primary neuronal cultures incubated with extracellular aSyn oligomers over time (n = 3-10; $P <$ 0.01, one-way ANOVA followed by a Dunnett's Multiple Comparison Test). (**e, f**) Representative immunoblots and quantification of the PrP^C and NMDAR2B levels in neuronal cultures incubated with extracellular aSyn oligomers over time (n = 6, n = 4; $P > 0.05$, one-way ANOVA followed by a Dunnett's Multiple Comparison Test). GAPDH was used as a loading control. GAPDH was used as a loading control. (**g**) Representative immunoblots and quantification of NMDAR subunit NR2B phosphorylation levels, normalized to NMDAR immunoreactivity, in neuronal cultures incubated with extracellular aSyn oligomers over time. (n = 3-7; $P < 0.05$, one-way ANOVA followed by a Dunnett's Multiple Comparison Test). (**h**) Quantitative analysis of IP:aSyn and IP:PrP^C (n = 3; means ± s.e.m., $P < 0.05$, two-sided unpaired t test). (**i**) Immunohistochemistry in 1.5 µm hippocampal sections from WT and aSyn Tg mice. aSyn is labelled in red and PrP^C is labelled in green (scale bar: 25 µm). At the bottom, details from the 63x magnification images are presented (scale bar: 5 µm).



Supplementary Figure 3.4. mGluR5 mediated aSyn/PrP^C long-term potentiation impairment. (**a**) Plot of the LTP magnitude (change in fEPSP slope at 50–60 min after theta-burst stimulation, compared to baseline) from control WT hippocampal slices (CTR, *n* = 4), slices pre-incubated with the mGluR5 antagonist MPEP (110 min, 5 µM, *n* = 3) and slices pre-incubated with extracellular aSyn oligomers alone (aSyn olig, 90 min, 500 nM, *n* = 6) or in the presence of MPEP (MPEP + aSyn olig, *n* = 4) (means ± s.e.m., $P < 0.05$, one-way ANOVA followed by a Bonferroni's Multiple Comparison Test). (**b**) Changes in fEPSP slope induced by theta-burst stimulation recorded from WT rat hippocampal slices pre-incubated with extracellular aSyn oligomers alone (aSyn mon, 90 min, 500 nM, *n* = 6), in the presence of the selective $A_{2A}R$ antagonist SCH-58261 (110 min, 50 nM, SCH + aSyn olig, *n* = 3) and in the presence of SCH-58261 together with the mGluR5 agonist DHPG (110 min, 10 µM; SCH + DHPG + aSyn olig, *n* = 4). (**c**) Plot of the LTP magnitude represented in **b** and in **Fig. 3.4d** (change in fEPSP slope at 50–60 min after theta-burst stimulation, compared to baseline) (means ± s.e.m., $P < 0.01$, one-way ANOVA followed by a Bonferroni's Multiple Comparison Test).



Supplementary Figure 3.5. Characterization of Thy1-aSyn (aSyn Tg) overexpressing mice. (**a**) Representative western blot of independent experiments to evaluate aSyn levels in the hippocampus (HIP) and striatum (STR) of WT and aSyn Tg mice. GAPDH was used as a loading control. At the bottom quantification of aSyn immunoreactivity in relation to WT (n = 2-5; means ± s.e.m., $P < 0.05$, two-sided unpaired t test). (**b**) Top panels: compositional images of fluorescence immunohistochemistry of WT and aSyn Tg mice hippocampus (scale bar: 500 µm). aSyn is identified in red fluorescence and cell nuclei are stained with Hoechst in blue fluorescence. Bottom panels: maximum intensity projection images of z-stack taken at 63x magnification in the CA1 area of hippocampus (scale bar: 25 µm). At the right, details from the 63x magnification images are presented (scale bar: 5µm). aSyn is identified in red fluorescence and SNAP25 is labeled in green. (**c**) Representative western blot of independent experiments to evaluate TH levels

in the striatum of WT and aSyn Tg mice. GAPDH was used as a loading control. At the bottom the respective quantification of TH immunoreactivity in relation to WT (n = 4; means ± s.e.m., P > 0.05, two-sided unpaired t test). (**d**) Representative images of TH immunohistochemistry of WT and aSyn Tg mice coronal brain sections. At the bottom the respective quantification of TH immunoreactivity in relation to WT (n = 3-4; means ± s.e.m., P > 0.05, two-sided unpaired t test). (**e**) Representative western blot of 3 independent experiments to evaluate PrP levels in the hippocampus of WT, aSyn Tg, and *Prnp*$^{-/-}$ mice. GAPDH was used as a loading control. At the bottom the respective quantification of PrP immunoreactivity in relation to WT (n = 5-6; means ± s.e.m., P < 0.05, two-sided unpaired t test).

Chapter IV

General Discussion and Concluding Remarks

General Discussion and Concluding Remarks

Increasing life expectancy will inevitably lead to an increase in the incidence of neurodegenerative disorders such as PD, constituting an increasing social and economic burden (Dorsey et al., 2007). At the same time, current dopaminergic therapies of PD, while effective in the initial motor symptoms, are accompanied by a loss of drug efficacy, the onset of motor complications, a lack of effect on NMS, and a failure to modify disease progression. As so, new treatments targeting beyond the damage dopaminergic system that are able to tackle disabling non-motor symptoms such as cognitive impairments, and are effective in both the early and late stages of PD, are urgently required.

Based on this, $A_{2A}R$ antagonists are now being recognized as important targets for the treatment of PD. The interest of $A_{2A}R$ was further emphasised by their prominent role in aSyn-mediated neurotoxicity since aSyn-induced damage to striatal neurons was clearly reduced in $A_{2A}R$ KO mice (Kachroo and Schwarzschild, 2012). Although the involvement of $A_{2A}R$ have been investigated extensively in PD-related motor deficits and associated brain areas, less is known about the role of this receptors in synaptic dysfunction, cognitive deficits and the underlying molecular mechanisms involved.

The work developed during my PhD and reported in the present dissertation, aimed at clarifying the pathological mechanisms driven by aSyn and the involvement of $A_{2A}R$ on this toxicity and to reveal the importance of this interaction in the light of synaptic functioning and early cognitive deficits, in order to obtain data supporting the potential therapeutic actions of $A_{2A}R$ in PD and other synucleinopathies.

Current clinical studies seem to indicate that neuronal aSyn accumulation, followed by synaptic impairments, underlie the onset of PD motor symptoms and neuronal death. This suggests that the loss of synaptic

function, and not the neurodegeneration, constitutes a crucial neuropathological event in the brain of PD patients (Bellucci et al., 2016). On the other hand, $A_{2A}R$ are now recognized as having a prominent role in controlling synaptic plasticity at glutamatergic synapses, via NMDAR activation (Cunha et al., 2008). In accordance with this view, the results here reported show that aSyn-synaptic impairments are rescued in $A_{2A}R$ KO mice or upon $A_{2A}R$ blockade. This synaptic protection afforded by $A_{2A}R$ inhibition is due to the reestablishment of glutamate NMDAR overactivation. These observations are consistent with previous reports showing that pharmacologic or genetic modulation of $A_{2A}R$ can prevent neurotoxicity and the extent of neuronal damage in neurons affected by ischemia, hypoxia, stress, or β-amyloid exposure (Batalha et al., 2013; Canas et al., 2009; Cunha, 2005; Valadas et al., 2012).

In addition to the effects on early synaptic dysfunction, $A_{2A}R$ antagonists were also effective in preventing subsequent neuronal death in neuronal cultures exposed to aSyn oligomers, and not monomers of fibrils, supporting the idea that soluble oligomeric species constitute the most neurotoxic species (Lashuel et al., 2012). Moreover, we showed that activation of $A_{2A}R$ *per si*, triggered the same toxic effect as the aSyn. This raises the hypothesis that aSyn is leading to a toxic overactivation of $A_{2A}R$. In fact, a recent study showed that aSyn instigates aberrant $A_{2A}R$ signaling (Hu et al., 2016). This can result either from, overexpression of $A_{2A}R$ in these conditions or, alternatively, to an increase overactivation of the receptor. The fact that the overexpression of aSyn did not alter $A_{2A}R$ levels favours the latter hypothesis.

aSyn aggregation, synaptic dysfunction and consequent neuronal cell loss are key neuropathological hallmarks of synucleinopathies, but the precise molecular mechanisms through which $A_{2A}R$ contributes to a NMDAR-mediated aSyn synaptic dysfunction remains unknown.

Recently the cellular form of PrP, a membrane-anchored neuronal glycoprotein whose normal function is uncertain, was identified as a cell surface binding partner for β-sheet-rich protein aggregates prompting their toxic effects. This, together with the fact that PrP^C is involved in age-dependent behavioral abnormalities, memory impairment, and mediates Ca^{2+} influx via NMDAR raised the hypothesis that PrP^C could also act as a mediator of aSyn synaptotoxic effects. We now establish a previously undocumented link between aSyn and PrP^C, whereby extracellular aSyn interacts with the amino acid region 93-109 of PrP^C forming a complex at the post-synaptic density that mediates disruption of Ca^{2+} signaling and consequently synaptic plasticity, via mGluR5-Fyn-NMDAR2B activation. Either blocking or deleting PrP^C prevents the toxic activation of this signaling pathway by aSyn oligomers and, consequently, reestablishes Ca^{2+} homeostasis and synaptic plasticity.

The identification of this downstream mGluR5 pathway linking aSyn-PrP^C to Fyn-NMDAR and synaptic toxicity also sheds light on the mechanisms by which $A_{2A}R$ are able to rescue NMDAR-dependent synaptic impairment. In fact, $A_{2A}R$ and mGluR5 co-localize and functionally interact at glutamatergic hippocampal synapses. Specifically, $A_{2A}R$ exert both a facilitatory and a permissive role on mGluR5-mediated effects, namely on phosphorylation of NMDAR2B (Try1472) (Sarantis et al., 2015; Tebano et al., 2005, 2006). Our results come in line with this studies since, $A_{2A}R$ blockade is able to prevent aSyn-mediated Fyn phosphorylation which directly phosphorylates NMDAR2B at tyrosine 1472, the same phosphorylation site regulated by aSyn.

Importantly, we now report, for the first time, that the *in vivo* selective blockade of $A_{2A}R$ (KW-6002) rescues both cognitive and synaptic impairments observed in aSyn Tg mice, providing the crucial evidence that the toxic effects of aSyn are indeed modulated by downstream effectors of PrP^C (mGluR5/Fyn), as we described *in vitro*.

KW-6002, also known as istradefylline, is approved in Japan for the adjunctive treatment of motor deficits in PD and was shown to be particularly suited to target the CNS, based on its bioavailability, half-life, and brain penetration in animal studies.

Additionally, it is important to mention that $A_{2A}R$ blockade *per si* was able to normalize NMDAR2B and PrP^C levels, whose expression are increased in aSyn tg animals. This suggests that $A_{2A}R$ blockade could not only impacting on mGluR5 downstream pathways but also be able to directly interfere with PrP^C. But it still remains to be directly shown that the beneficial effects of blocking $A_{2A}R$ in aSyn-induced toxicity, are hampered or not by manipulation of PrP^C.

Another significant finding of our studies is that $A_{2A}R$ are able to interfere with the latter stages of aSyn aggregation process, since blockade/activation of $A_{2A}R$ leads, respectively, to a decrease/increase of cells containing aSyn aggregates. This can be either due to an alteration of aggregates formation or a modification in the proper aggregation clearance. Intracellular Ca^{2+} transient increase has been shown to induce cytoplasmic aSyn aggregates (Rcom-H'cheo-Gauthier et al., 2014). Having this in mind and knowing that $A_{2A}R$ blockade rescues NMDAR overactivation and consequently Ca^{2+} dysfunction, we might propose that the reestablishment of the mGluR5-NMDAR signaling, inhibits aSyn aggregation by Ca^{2+} buffering, supporting the first hypothesis.

While Ca^{2+} dysfunction contribute to aSyn aggregate formation, components of the proteostasis network dictate the fate of protein aggregates. Both alterations in the two major degradation systems, the ubiquitin-proteasome system (UPS) or the autophagy-lysosomal pathway (ALP) have been linked NMDAR activation and aSyn pathology (Caldeira et al., 2013; Ebrahimi-Fakhari et al., 2011). Furthermore, there are also reports suggesting that $A_{2A}R$ can directly bind and modulate the activity of UPS (Milojevic et al. 2006; Chiang et al. 2009), supporting the second

hypothesis. Taken together these suggests that blocking $A_{2A}R$, and consequently establishing NMDAR-Ca^{2+} dysfunction, might prevent both aSyn aggregation formation and aSyn aggregates accumulation, by rescuing UPS and ALP dysfunction. Although we cannot exclude the involvement of downstream mediators of aSyn toxicity.

Moreover, these data strongly suggest that the recently reported ability of caffeine, a nonselective adenosine receptor antagonist, to interfere with aSyn aggregation might be due to its actions on $A_{2A}R$ (Kardani and Roy, 2015). Again, whether these effects are due to the attenuation of UPS and ALP dysfunction or to downstream mediators of aSyn toxicity remains to be clarified.

Overall, our study sheds light into the early pathophysiological mechanisms preceding aSyn-mediated neurodegeneration, and implicates $A_{2A}R$ and PrP^C as key molecular target in PD and other synucleinopathies (**Fig. 4.1**). Furthermore, the ability of the *in vivo* $A_{2A}R$ blockade to restore synaptic impairments and cognitive deficits in aSyn Tg mice, in parallel to studies linking $A_{2A}R$ to cognitive function and neurodegeneration, provides an additional mechanistic support to encourage testing the therapeutic efficacy of $A_{2A}R$ antagonists in early PD patients.

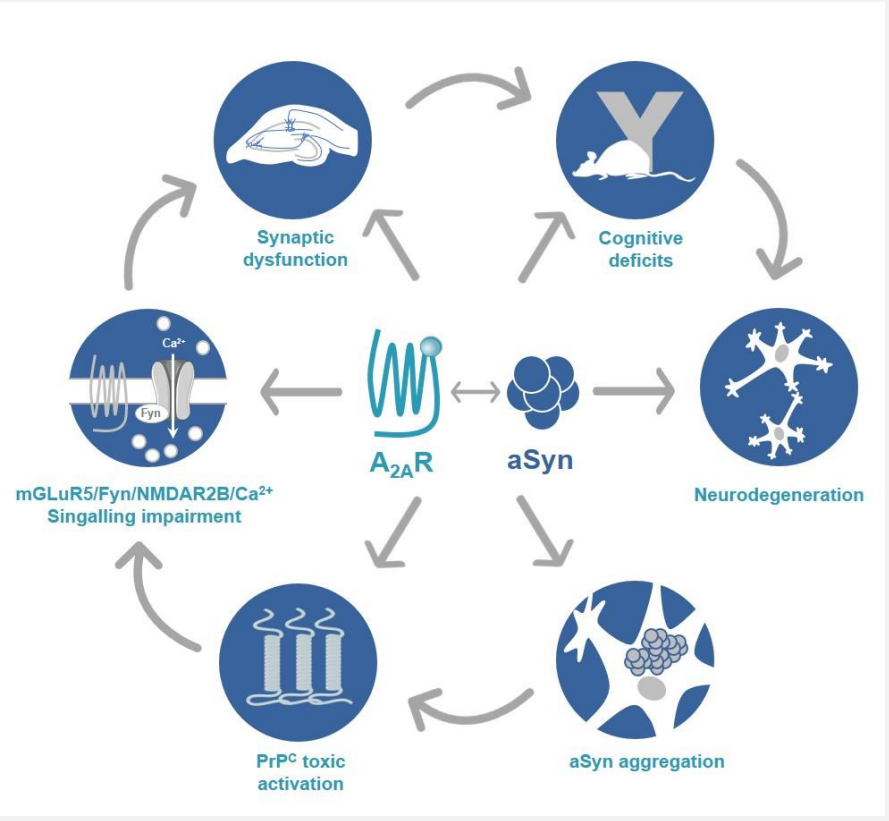


Fig. 4.1 Schematic representation of aSyn pathological mechanisms mediated by $A_{2A}R$. In pathological conditions where aSyn levels are exacerbated and aSyn aggregation occur, there is an impairment of the Fyn-NMDAR signaling and as a consequence, there a toxic increase in intracellular Ca^{2+} levels, which in turn mediate synaptic dysfunction, associated with cognitive deficits, and eventually, at latter phases, neuronal death. This is accompanied by an activation of PrP^C and $A_{2A}R$, both contributing to a toxic Fyn-NMDAR signaling via mGLuR5, explaining the rational of blocking $A_{2A}R$ to prevent synaptic toxicity and neurodegeneration. When $A_{2A}R$ are blocked, Fyn-NMDAR2B signaling is reestablished, resulting in restored hippocampal function, memory performance and synaptic plasticity. One the other hand, $A_{2A}R$ blockade is also able to restore PrP^C level and interfere with aSyn aggregation.

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– Antoine de Saint- Éxupery

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– Antoine de Saint- Éxupery

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Pelo sonho é que vamos,
comovidos e mudos. (...)
Chegamos? Não chegamos?
– Partimos. Vamos. Somos
– Sebastião da Gama

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Appendix

Reprint of published papers